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**The humoral response against *Salmonella* Typhi  
protein antigens during acute, convalescent,  
and chronic typhoid fever**

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A thesis submitted to the Open University UK

For the degree of Doctor of Philosophy in the field of Life Sciences

Oxford University Clinical Research Unit

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## **Abstract**

Enteric (typhoid) fever is a life-threatening disease caused by the *Salmonella enterica* subspecies *enterica* serovars Typhi (*S. Typhi*) and Paratyphi A, B, and C (*S. Paratyphi* A, B, and C). The disease still causes major public health problems in low- and middle-income countries, principally in Asia and Africa. The increasing frequency of multi-drug resistant (MDR) and extended-drug resistant isolates (XDR) of *S. Typhi* and an increasing incidence of *S. Paratyphi* A mean that the international dynamics of enteric fever are changing. These changes add urgency to the demand for more efficient enteric fever control campaigns. The aim of this thesis was to assess control measures for enteric fever in Vietnam and to develop techniques that can be used as further control methods. I firstly systemically reviewed retrospective information regarding enteric fever in Vietnam and combined these data with data on economic development. This investigation revealed that national economic growth, the provision of improved quality drinking water, and better sanitation were likely the greatest contributors to the decline and ultimate elimination of enteric fever in Vietnam. My work then evaluated the serodiagnostic potential of a panel of novel *S. Typhi* protein antigens and the Vi capsular polysaccharide (Vi) in a group of patients with febrile diseases in Bangladesh. These data demonstrated the utility of serology for typhoid diagnostics when exploiting a combination of Vi and at least one protein antigen. I then assessed the acquisition of antibody against typhoid toxin during natural *S. Typhi* and *S. Paratyphi* A infections and measured the capability of these antibodies to neutralise the toxin. The data provided supporting evidence for generating an antitoxin treatment for enteric fever (caused by both *S. Typhi* and *S. Paratyphi* A), and potentially encourages the use of typhoid toxin in vaccine formulations. Within the scope of searching for vaccine novel candidates, my work

further identified a panel of immunogenic antigens shared between *S. Typhi* and *S. Paratyphi A* that can stimulate an antibody response which can instigate bactericidal killing during natural infection. Finally, by exploiting the unique immunological profiles of *S. Typhi* carriers (cytokines and antibody), I developed a method of identifying *S. Typhi* carriers and estimating the prevalence of *S. Typhi* carriage in a typhoid endemic population. My findings will potentially lead to the development of novel enteric fever control strategies. I conclude that improved case detection and widespread vaccination campaigns using polyvalent *Salmonella* vaccines should be initiated for reducing the burden of enteric fever in endemic areas.



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## **Declaration**

I, Tran Vu Thieu Nga, declare that the majority of the data presented in this thesis was derived from my own work. Due to the nature of these studies and the interdisciplinarity nature of biological sciences, it was not possible to conduct all of the methods in this thesis; however, I did design all of the experiments generating the data for this thesis. The details of the various contributors are clearly stated in the Materials and Methods.

## Abbreviations

µg	Microgram
µM	Micromole
µl	Microliter
Ω	Ohms
AC	Afebrile control
AIDS	Acquired Immune Deficiency Syndrome
AMR	Antimicrobial resistance
ATP	Adenosine triphosphate
Bp	Base pairs
BRC	Baby rabbit complement
BSA	Bovine serum albumin
CFU	Colony Forming Unit
DNA	Deoxyribose nucleic acid
dMT	Descendant mutant (strain)
dNTPs	Deoxyribonucleotide triphosphates
EDTA	Ethylene-diamine-tetra-acetic acid
ELISA	Enzyme Linked Immunosorbent Assay
EU	ELISA Unit
Kg	Kilogram
kV	kilovolt
LMIC	Low- and middle-income country
LPS	Lipopolysaccharide
MDR	Multi-drug resistant
mF	Millifarad
mg	Milligram
mL	Millilitre
NaOAc	Acetic acid sodium salt
nm	Nanometre
OD	Optical density
PBS	Phosphate buffered saline
PCA	Principal component analysis
PCR	Polymerase chain reaction
RDT	Rapid diagnostic test
ROC	Receiver operating characteristic
Rpm	Rounds per minute
SBA	Salmonella bactericidal assay
SVM	Support vector machine
TE	Tris EDTA buffer
TraDIS	Transposon Directed-Insertion Site Sequencing
WT	Wild type (strain)
SPI	<i>Salmonella</i> Pathogenicity Island
ST	<i>Salmonella</i> Typhi
SPA	<i>Salmonella</i> Paratyphi A
UF	Unidentified pathogen febrile

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## Chapter 1 Introduction

### 1.1 Introduction to enteric fever

Typhoid fever (typhoid) is thought to be one of the oldest human diseases for which there are written records, and probably caused the plague of Athens in 430 B.C. <sup>1</sup>, but the disease was not clearly distinguished from rickettsial disease (typhus) until the mid-nineteenth century. The name “typhoid” implies a “typhus-like” condition and ‘typhus’ is derived from the Greek word for cloud, associated with the fact that typhoid/typhus patients have a clouded brain as they can be delirious <sup>2</sup>. Typhoid is a systemic life-threatening human disease caused by the bacterium *Salmonella enterica* subspecies enterica serovar Typhi (*S. Typhi*) <sup>3</sup>. Paratyphoid, caused by the *Salmonella enterica* subspecies enterica serovars Paratyphi A, B, and C (*S. Paratyphi A* etc.) is a disease that is clinically indistinguishable from typhoid <sup>4</sup>, collectively typhoid and paratyphoid are referred to as enteric fever. The various *Salmonella* serovars that cause enteric fever are distantly related to one another, have arisen to cause a similar infection through convergent evolution <sup>5,6</sup> and are referred to as “typhoidal salmonellae” <sup>7</sup>. The organisms that cause enteric infections are transmitted via contaminated food or water. Consequently, enteric fever remains a substantial public health issue in large cities and rural populations that have poor sanitation and limited access to safe water <sup>8–10</sup>. Therefore, the vast majority of enteric fever cases now occur in countries in south-central Asia, southeast Asia and Africa <sup>11</sup>.

## **1.2 *Salmonella* Typhi and *Salmonella* Paratyphi A**

### **1.2.1 Taxonomy**

*S. Typhi* and *S. Paratyphi A* are in the large bacterial genus *Salmonella*, which falls within the largest Gram-negative bacterial family, the *Enterobacteriaceae*. Current classification segregates the genus *Salmonella* into two species, *Salmonella enterica* and *Salmonella bongori* <sup>12</sup>. *Salmonella enterica* (*S. enterica*) is comprised of six subspecies: *enterica* (I), *salamae* (II), *arizonae* (IIIa), *diarizonae* (IIIb), *houstenae* (IV), and *indica* (VI). These six subspecies are further divided into approximately 2,500 different serovars based on their O (lipopolysaccharide) and H (flagella) antigens; *S. Typhi* and *S. Paratyphi A* are found with majority of other *Salmonella* in subspecies *enterica* <sup>12</sup>.

### **1.2.2 Bacteriology of *Salmonella* Typhi and *Salmonella* Paratyphi A**

When *S. Typhi* or *S. Paratyphi A* are isolated from clinical specimens they form smooth, round colonies on nutrient agar. On Kligler iron agar they can be primarily identified by the characteristic biochemical pattern of producing acid without gas, and are moderate H<sub>2</sub>S producers on an alkaline slant <sup>3</sup>. The general method for identifying *Salmonella* in clinical microbiology laboratories is serotyping following the Kauffmann-White scheme. *Salmonella* serotyping is performed by assessing antibody agglutination reactions with the bacterial surface structures, which includes the O-antigen (somatic, lipopolysaccharide) and H-antigen (flagella). According to the Kauffmann-White scheme, *S. Typhi* belongs to serogroup O9 or group D (type O9 and 12), while *S. Paratyphi A* belongs to into serogroup O2 or group A (type O1, O2 and O12). In addition, *S. Typhi* identification is also confirmed by the serological demonstration of the Vi (virulence) capsular polysaccharide.

*S. Typhi* and *S. Paratyphi A* are exclusively human restricted pathogens <sup>13</sup>. They have evolved convergently to possess many shared characteristics, consequently their pathological and epidemiological lifestyles are highly comparable <sup>4,14</sup>. The course of disease associated with infection by these two typhoidal *Salmonella* serovars are largely indistinguishable, yet they differ substantially from the disease caused by the non-typhoidal *Salmonella* serovars <sup>14</sup>. Notably, the establishment of a chronic infection in the human gallbladder is another feature shared between the two serovars that differs from the non-typhoidal *Salmonella* serovars <sup>15</sup>. Transmission of *S. Typhi* and *S. Paratyphi A* occurs via the faecal-oral route, which arises mainly through the consumption of contaminated water and food. Furthermore, *S. Typhi* and *S. Paratyphi A* infections can also be spread via person to person to cause epidemics <sup>16</sup>. A further shared characteristic between *S. Typhi* and *S. Paratyphi A* is that they are clonal, and the populations of both organisms are monophyletic <sup>17</sup>. Individual genomes of *S. Typhi* and *S. Paratyphi A* are highly conserved and have limited evidence of recombination and horizontal gene transfer <sup>17,18</sup>.

### **1.2.3 Enteric fever**

#### **1.2.3.1 The clinical features of enteric fever**

A common misconception is that *S. Paratyphi A* causes a less severe disease with fewer complications than *S. Typhi* <sup>12</sup>; however, in the largest comparison of infections caused by both *S. Typhi* and *S. Paratyphi A* to date, the clinical features of both organisms were indistinguishable in terms of clinical presentation, duration, and outcome <sup>4</sup>. Enteric fever has a well-studied disease progression in both natural infection and human challenge. The first disease symptoms appear 8 to 14 days

(range 3-60 days) after ingestion of the bacteria <sup>3</sup>, which marks the onset of the major period of bacteraemia. The temperature progressively rises and is maintained at 39 to 40°C by the second week of disease. Typical symptoms include prolonged fever and malaise. Enteric fever is also accompanied by various symptoms in the acute phase such as flu-like discomfort (chills and frontal headache), anorexia, nausea, dry cough, and bowel pain. Some patients may also have a coated tongue, hepatomegaly, and splenomegaly <sup>16</sup>. Several immunologic symptoms such as leukopenia, and neurological complications can also be commonly observed <sup>16</sup>. The clinical presentation and disease severity of enteric fever can widely vary due to several factors including the length of illness prior to receiving appropriate treatment, the virulence and antimicrobial susceptibility profile of the infecting organism, appropriate antimicrobial therapy, the age of the patient, previous exposure, vaccination profile, inoculum, and other host factors (i.e. AIDS or other immune suppression) <sup>19-24</sup>. The occurrence of minor complications and severe complications can also vary greatly and can be dependent on the medical care in the healthcare facility in which the patient is admitted. Neuropsychiatric manifestations can also occur in enteric fever, again the prevalence of such complications is highly variable depending on the study and may arise in 5% to 84% of typhoid patients <sup>25-28</sup> at the acute phase or after the initial fever has subsided <sup>29</sup>. Other complications have been reported, such as gastrointestinal bleeding and intestinal perforation, which are often associated with poor outcome, including death <sup>30</sup>.

More recently (the end of the last century onwards), severe and fatal typhoid has been substantially reduced to <1% in most places where effective antimicrobial treatments are available <sup>31</sup>. Notably, these figures ranged from 7 to 26% of all

hospitalized cases prior to the introduction of antimicrobials <sup>13,31</sup>. Severe enteric fever has been reported to occur in very young children and elderly, and patients not receiving adequate antimicrobial therapy <sup>22,32</sup>. A study conducted in Vietnam during 1993 to 1995 and 1997 to 1999 reported that severe typhoid was associated with increasing age and intermediate susceptibility to ciprofloxacin but not MDR phenotype <sup>23</sup>. Symptomatic disease relapse can occur in both antimicrobial treated and untreated patients at a rate of 5 to 10% of all cases. However, relapse appears to occur at a lower rate in patients with susceptible infections treated with newer generation antimicrobials including second generation fluoroquinolones (1.5%) or third generation cephalosporins (5%) in comparison to those treated with traditional first line drugs (chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin) <sup>33–38</sup>. Isolated organisms from relapse cases typically have the same antimicrobial susceptibility pattern as the ones identified from the original episode. The organisms causing relapse can be discriminated from those causing reinfection by molecular typing <sup>39</sup>.

### **1.2.3.2 The treatment of enteric fever and antimicrobial resistance**

Enteric fever patients in endemic areas are not always treated in hospital, rather they self-manage at home with oral antimicrobials and may be followed-up regularly for complications <sup>40,41</sup>. Generally, only a small number of enteric fever patients are hospitalized, limited to those with more severe symptoms. For these patients, it is critical they are clinically managed to prevent serious complications and death. Such provisions include the timely administration of effective antimicrobials, good nursing care, the maintenance of appropriate nutrition and hydration. Even though the use of appropriate antimicrobials has been proven to be the most effective



therapy to reduce mortality, complications, and duration of the illness <sup>3</sup>, the selection of antimicrobial is not always straight forward. The choice of drugs in endemic areas is based on a variety of important principles such as their availability, efficacy, and cost <sup>42</sup>.

The emergence of antimicrobial resistant enteric fever infections has become a major concern in many endemic areas <sup>32,43–49</sup>. Until the mid-1970s, chloramphenicol was the chief first-line drug for enteric fever treatment. Chloramphenicol resistant outbreaks of *S. Typhi* arose in Mexico and India in the 1970s <sup>49</sup>. In the following years outbreaks occurred in Pakistan, India, and Bangladesh, and the organisms had acquired a multi-drug resistance (MDR) plasmid which induced resistance to the three primary antimicrobials (ampicillin, chloramphenicol, and co-trimoxazole) <sup>50,51</sup>. The introduction of fluoroquinolones became essential after the failure of chloramphenicol and the emergence of MDR organisms. However, reports of *S. Typhi* and *S. Paratyphi A* that are resistant to nalidixic acid and exhibit reduced susceptibility to fluoroquinolones in different parts of Asia have become more prevalent <sup>43,46,50</sup>. These organisms became associated with rising frequencies of fluoroquinolone treatment failure <sup>33,52–54</sup>. Effective alternative antimicrobials for treating *S. Typhi* and *S. Paratyphi A* with reduced susceptibility to fluoroquinolones include the third generation cephalosporins (i.e. ceftriaxone, cefixime, cefotaxime, and cefoperazone) and the macrolide azithromycin <sup>52,55–61</sup>. Alternatively, the original first line drugs (ampicillin, chloramphenicol, and co-trimoxazole) can still be used effectively in regions where the circulating organisms remain susceptible to these antimicrobials. Regarding the treatment of carriage, some reports have indicated a prolonged course of the same antimicrobials used for acute infections <sup>62,63</sup>. However,

various antimicrobial regimes have been suggested to treat typhoid carriage yet none have been demonstrated to be completely effective in the eradication of the chronic colonized pathogens <sup>64–66</sup>.

### **1.3 The epidemiology of typhoid fever**

The epidemiology of enteric fever changed substantially in the twentieth century, facilitated by regional population growth and delivery of clean water and better sanitation systems <sup>67</sup>. Historically, enteric fever caused by *S. Typhi* had been largely considered more common than *S. Paratyphi A*. However, disease caused by *S. Paratyphi A* has become increasingly prevalent in many low- and middle-income countries (LMICs) in south and southeast Asia <sup>68–70</sup>. An increased incidence of *S. Paratyphi A* enteric fever has been reported in India, Pakistan, China, Nepal, and Cambodia <sup>69,71,72</sup>. An annual increase of *S. Paratyphi A* enteric fever was reported in a 10-year surveillance study of blood cultures in Kathmandu, Nepal; the overall incidence rate of *S. Typhi* enteric fever remained unchanged <sup>73</sup>.

The global estimate of enteric fever disease burden is often adjusted for the poor sensitivity (50 to 60% <sup>74</sup>) of blood culture <sup>67,75</sup> and/or water-related risk <sup>76</sup>.

Accordingly, with adjustment for poor sensitivity of blood culture, in 2000, *S. Typhi* was estimated to cause 21.6 million new cases and 216,500 deaths globally per year (crude estimate of 10.8 million cases); the equivalent figure for *S. Paratyphi A* was adjusted to an estimate of 5.4 million cases <sup>67</sup>. Ten years later (in 2010), for the same adjustment method, the global burden of *S. Typhi* disease was of 26.7 million cases (crude estimate, 13.5 million cases) <sup>75</sup>. However, the global burden of enteric fever

in 2010 was estimated to be lower, with 11.9 million cases and 129,000 deaths annually when adjusted for water-related risk and using data from LMICs <sup>76</sup>.

The most recent systemic review on the global burden of enteric fever in 2017 provided estimates of 14.3 million episodes of enteric fever of which typhoid was responsible for 76.3% of all cases <sup>77</sup>. Enteric fever in non-endemic areas in developed countries is rare, but unvaccinated travellers visiting areas where enteric fever is endemic are at risk of contracting the disease. There are approximately 400 reported cases of enteric fever in the United States and the United Kingdom annually, the majority of these are associated with a recent travel history to south Asia <sup>78,79</sup>.

### **1.3.1 Enteric fever in Asia**

The majority of epidemiological studies for enteric fever have been performed in Asia; therefore, Asia is considered the main global hub for enteric fever. More than 90% of the reported morbidity and mortality associated with enteric fever in 2000 occurred in Asia <sup>80</sup>. In this year, the incidence of *S. Typhi* associated enteric fever in Asia was estimated to be 394.2/100,000 population <sup>67</sup>. Despite the common circulation of MDR organisms and the emergence of fluoroquinolone resistance in many parts of Asia, there has been an encouraging reduction in the burden of enteric fever across Asia; this is particularly valid in locations where there have been improvements in clean supply and sanitation. A decline of 72 to 6.2% of *S. Typhi* positive blood cultures over 15 years from 1998-2004 was reported in the south of Vietnam <sup>81</sup>.

### **1.3.2 Enteric fever in Africa**

Enteric fever has become recognized as a major cause of febrile disease in many parts of Africa over the last decade despite the fact that the disease burden is probably under-estimated because of an overlap with the symptoms of malaria <sup>82</sup>. A community acquired bloodstream infection study conducted in Nigeria in 2008 and 2009 found that 55% of children with a fever receiving a blood culture were confirmed to have *S. Typhi*, but were clinically diagnosed with malaria <sup>83</sup>. *S. Typhi* was responsible for 9.9% of all culture positive bloodstream infections across Africa during 1984 - 2006 <sup>82</sup>. Notably, a fever study conducted in Zanzibar found that *S. Typhi* comprised 58% of all bacterial pathogens isolated from bloodstream infections (March 2009 till December 2010) <sup>84</sup>. This isolation rate was considered high and may reveal typhoid incidence that are as comparably high as those recorded in regions in Asia. Moreover, the systemic review on global burden of enteric fever in 2010 reported that the highest incidence rate was in sub-Saharan Africa (724.6/100,000 people) <sup>75</sup>.

### **1. 4 Enteric fever risk factors**

Risk factors for contracting enteric fever include drinking contaminated water, consuming food prepared outside the home, and poor housing with inadequate personal hygiene. The transmission of *S. Typhi* could be also associated with contact with a recently infected individual who may be shedding infectious organisms <sup>85,86</sup>. Other reported risk factors for *S. Typhi* infection include flies, laboratory accidents, unsterile medical instruments, and oral-anal intercourse <sup>13</sup>.

The vast majority of enteric fever cases occur in school-age children (5-16 years) and young adults <sup>87,88</sup>. However, in some settings, typhoid fever is also highly prevalent in children <5 years of age <sup>89</sup>. Additionally, outbreaks of enteric fever involving all age groups can occur in the rapidly expanding conurbations of Asia, where sanitary conditions and a lack of hygiene permit the transmission of the organisms <sup>3</sup>.

The health status of the individual is also an important factor for enteric fever susceptibility. A case-control study conducted over a period of one year in an urban area in India reported that enteric fever cases were associated with the detection of anti-*Helicobacter pylori* antibodies; the presence of anti-*Helicobacter pylori* antibodies was associated with enteric fever with an adjusted odds ratio of 2.03 (1.02–4.01) <sup>24</sup>. This observation supported a report on vaccinees immunized with the live oral vaccine CVD 908-*htrA*; individuals infected with *H. pylori* had significantly elevated *S. Typhi*-specific IgG LPS than those uninfected with *H. pylori* <sup>90</sup>.

There have been only a modest number of studies regarding the influence of human genetic factors on the variation of enteric fever in terms of incidence and disease severity, this is in spite of the enormous number of genetic associations found in mice with respect to *Salmonella* susceptibility. From the studies that have been performed, very few human genetic factors have been found to contribute to enteric fever susceptibility. In the studies that have identified genetic factors associated with susceptibility to enteric fever, genes involved in the immune response to disease appear to be pivotal. Human major histocompatibility complex (MHC) class II and

III genes were found to associate with *S. Typhi* enteric fever investigated on patients from two geographic locations in the south of Vietnam <sup>19</sup>. Accordingly, HLA-DRB1\*0301/6/8, HLA-DQB1\*0201-3, and TNFA\*2(-308) were associated with susceptibility to typhoid fever, while HLA-DRB1\*04, HLA-DQB1\*0401/2, and TNFA\*1(-308) appeared to be associated with disease protection <sup>19</sup>. A genome-wide association study from the same author conducted with a large collection of 432 cases and 2,011 controls from Vietnam, which was replicated with a collection from a Nepali cohort and a second independent collection from Vietnam, confirmed their role as protective factor against enteric fever for HLA-DRB1\*04:05 <sup>91</sup>. Toll-like receptors (TLRs) mediate the innate immune responses to bacterial pathogens; TLR5 is stimulated by bacterial flagellin, whilst TLR4 is triggered by LPS. Genetic variation within TLR4 was found to be potentially protective for enteric fever in a Vietnamese population <sup>92</sup>, polymorphisms within the genes encoding TLR5 were found not to play a significant role in TLR-stimulated innate immune responses to *S. Typhi* infection <sup>93</sup>. A more recent genome-wide association study conducted in a Vietnamese population found that raised VAC14 (a phosphoinositide-regulating protein) expression increased plasma membrane cholesterol, which assisted *Salmonella* invasion, resulting in increased susceptibility to enteric fever <sup>94</sup>. The cystic fibrosis transmembrane conductance regulator (CFTR) is the affected protein in cystic fibrosis (CF) expressed on the intestinal mucosa. *S. Typhi* uses CFTR to invade intestinal epithelial cells <sup>95</sup>. A study conducted in a typhoid endemic area in Indonesia genotyped two highly polymorphic markers in CFTR and the most common CF mutation, showed an association between genotypes in CFTR and susceptibility to typhoid fever (OR=2.6) <sup>96</sup>.

## **1.5 Virulence factors of *Salmonella* Typhi and *Salmonella* Paratyphi A**

There is approximately 10% difference in the gene content of *S. Typhi* and *S. Typhimurium*; approximately 480 genes are unique to *S. Typhimurium* <sup>97</sup> and >600 genes are unique to *S. Typhi* <sup>98</sup>. Much of this variation makes those two comparatively closely related bacteria induce two distinct infectious diseases in humans <sup>99</sup>. The genetic differences found in *S. Typhi* include plasmids, pathogenicity islands, and the formation of pseudogenes. A combination of these factors contribute to the distinct disease *S. Typhi* causes <sup>97,99–101</sup>.

### **1.5.1 Plasmids**

A global analysis of *S. Typhi* isolates revealed that the *S. Typhi* population is highly clonal with limited genomic variation <sup>18,102</sup>. Accordingly, this serovar seldom carries plasmids and the self-transmissible IncH1 group is the only commonly found plasmid in *S. Typhi* <sup>103</sup>. The IncH1 plasmid is associated with an MDR phenotype and commonly carries the AMR genes *cat*, *dhfr7*, *dhfr14*, *sul1*, and *bla*<sub>TEM-1</sub> <sup>104</sup>. The presence of these IncH1 plasmids has been associated with disease severity and fatality <sup>105</sup>.

Plasmid pSLT, is a self-transmissible virulence plasmid, harboured by the majority of *S. Typhimurium* isolates, which contains the *spv* operon <sup>106</sup>. The *spv* locus has been found to facilitate serum resistance in *S. Typhimurium* and also enhance the ability of the organism to adhere, colonize, survive, and replicate in host cells <sup>107,108</sup>. This plasmid (and operon) is notably absent from *S. Typhi*. However, a recent study found a homologue of the *spv* operon locus with 99.8% sequence identity on an alternative plasmid (pR<sub>ST98</sub>) in *S. Typhi* <sup>109</sup>. This plasmid was isolated from *S. Typhi*

associated with an outbreak in China in the 1980s <sup>110</sup>. AMR genes (ampicillin, chloramphenicol, carbenicillin, cephalosporin, gentamicin, kanamycin, neomycin, streptomycin, sulphonamide, trimethoprim and tetracycline) and virulence genes were located on pR<sub>ST98</sub>, making it a hybrid resistance-virulence plasmid <sup>110</sup>. *In vitro* studies of pR<sub>ST98</sub> found that the presence of this plasmid impairs the dendritic cell response to infection <sup>110</sup>, enhances intracellular bacterial growth <sup>109</sup>, and impairs the autophagic flux in infected macrophages <sup>111</sup>.

Lastly, pBSSB1, a linear plasmid, was described in *S. Typhi* isolates from Indonesia and was found to carry the *fljB<sup>z66</sup>* gene. The product of the *fljB<sup>z66</sup>* gene is an alternative flagellin antigen known as H:z66. The association between the presence of this plasmid and pathogenesis of *S. Typhi* still remains largely unknown <sup>112</sup>.

### **1.5.2 *Salmonella* pathogenicity islands (SPIs)**

The virulence-associated genes in *Salmonella* genomes are typically clustered together in pathogenicity islands. Among known 21 *Salmonella* pathogenicity islands (SPIs), only one SPI-14 is specific for *S. Typhimurium* and *S. Typhi* possess 17 SPIs, 11 are shared between *S. Typhi* and *S. Typhimurium* (1-6, 9, 11, 12, 13, and 16), four (SPI-7, -15, -17, -18) are unique to *S. Typhi* <sup>97</sup>. SPI-1 and SPI-2 are well described and each encodes a type three secretion system (TTSS) <sup>97</sup>. Over 40 effector proteins are associated with these two TTSSs and are secreted through the needle complex to manipulate the host cell during infection. The SPI-1 TTSS secretes effectors, which assist extracellular *Salmonella* to invade nonphagocytic host cells (such as M cells) and induce pro-inflammatory activation <sup>113</sup>. A recent study comparing the bile responses of *S. Typhi* and *S. Typhimurium* revealed that



upon exposure to bile there is significant up-regulation of SPI-1 genes in *S. Typhi* but not *S. Typhimurium* <sup>114</sup>. Alternatively, whilst the SPI-2 TTSS is crucial for the proliferation of *S. Typhimurium* within the macrophage *Salmonella*-containing vacuole (SCV) <sup>115</sup>, *S. Typhi* does not rely on SPI-2 TTSS to survive in human macrophages <sup>116</sup>.

### **1.5.3 Flagella and fimbriae**

Flagellum is a complex structure that consists chiefly of the flagella filament, of which the major constituent is polymerized flagellin. Flagella enable bacterial motility and are an essential antigen that triggers innate immune responses <sup>117</sup>. Expression of *S. Typhi* flagellin has been demonstrated to be partially down regulated by TviA, (a transcriptional regulator associated with the *viaB* locus on SPI-7 in *S. Typhi*; notably this regulator is generally absent in *Salmonella* serovars that cause gastroenteritis) <sup>118</sup> during invasion of intestinal epithelial cells <sup>119</sup>. This down regulation subsequently enables *S. Typhi* to subvert the innate immune system by limiting inflammasome activation in infected phagocytes. As previously mentioned, under an experimental culture conditions supplemented with 3% bile, *S. Typhi* but not *S. Typhimurium* up-regulates SPI-1 gene expression and flagellin production <sup>114</sup>. This observation may explain the contribution of the flagellar filament to *S. Typhi* biofilm formation on gallstones <sup>120</sup>.

For *S. Typhi* to initiate an infection it requires to colonise the host and needs to adhere to cells, which is assisted by various adhesins <sup>121</sup>. Fimbriae and pili are important adhesion factors for *S. Typhi*. These virulence factors are employed by *S. Typhi* for its various cellular interactions during infection and host colonization <sup>122</sup>.

The *stg* operon, one of the six fimbriae operons that are found in *S. Typhi* but not *S. Typhimurium*, has been recently demonstrated to be involved in cellular invasion and epithelial cell disruption *in vitro* <sup>122</sup>. Furthermore, the *stg* operon was found to aid *S. Typhi* in preferentially targeting enterocytes than M cells, which facilitates *S. Typhi* to elude the innate immune system by bypassing the Peyer's patches <sup>123</sup>.

#### **1.5.4 The Vi capsular polysaccharide**

*S. Typhi* is capsulated by the Vi (virulence) polysaccharide, which is indistinguishable from those found in *S. Paratyphi C* and some strains of *S. Dublin* and *Citrobacter freundii* <sup>118</sup>. The Vi capsular polysaccharide is a homopolymer of  $\alpha$ -1,4(2-deoxy)-2-N-acetyl-3-O-acetylgalacturonic acid, which does not contain free hydroxyl groups <sup>118</sup>. Vi was first purified from a fresh culture of *S. Typhi* in 1943 and has been studied extensively. The *ViaB* locus, which is located on SPI-7, is a cluster of 10 genes which are responsible for regulation (*tviA*), biosynthesis (*tviBCDE*) and export (*vexABCDE*) of the Vi capsule <sup>118</sup>. Despite the isolation of Vi-negative *S. Typhi* variants <sup>124,125</sup>, these organisms are rare, which suggests that this antigen is a vital characteristic of the organism <sup>126</sup>. As an enteric pathogen, after entering the gastrointestinal tract, *S. Typhi* experiences a range of harsh environmental conditions, including raised temperature, low pH and high pH, reduced oxygen abundance, poor nutrition, and mixed osmolarity <sup>127</sup>. Notably, osmolarity is one of the principal factors that regulates the production of Vi through *tviA* <sup>128–130</sup>.

Several facts support the non-essential role of Vi for pathogenicity including the isolation of Vi-negative *S. Typhi* variants from blood <sup>131</sup>, and the ability of other Vi

negative *Salmonella* serovars to cause enteric fever <sup>4</sup>. However, Vi is largely considered as a crucial virulence factor for *S. Typhi*. Various experimental studies have highlighted the importance of Vi in *S. Typhi*; these include: i) the lethal dose of *S. Typhi* is reduced by 50% when mice are infected with Vi negative organisms, compared to Vi positive organisms <sup>132</sup>, ii) Vi deficient *S. Typhi* is less virulent than Vi positive *S. Typhi* in human volunteers <sup>132</sup>, iii) Vi assists serum resistance to non-Vi antibodies, prevents complement deposition, and phagocytic opsonization <sup>133</sup>. Furthermore, clinical evidence suggesting Vi is an essential *S. Typhi* virulence factor includes: i) clinical *S. Typhi* from typhoid patients are predominantly Vi-positive <sup>13,133</sup>; ii) licensed Vi subunit vaccines have been widely demonstrated to confer comparable protective efficacy to the licensed live oral attenuated *S. Typhi* vaccines <sup>134,135</sup>.

### **1.5.5 Typhoid toxin**

Amongst the most interesting loci among the genomic regions unique to *S. Typhi* compared to *S. Typhimurium* is the one encoding typhoid toxin, the CdtB-islet. This small region comprises of genes encoding three components of typhoid toxin including CdtB (cytolethal distending toxin B serving nuclease activity), pltA (ADP-ribosyltransferase homologous to the S1 subunit of the pertussis toxin), and pltB (another pertussis like toxin) as well as other two proteins encoding genes responsible for its secretion and transportation. Typhoid toxin is an A2B5 toxin composing 2 active subunits linked by a disulfide bridge (CdtB and PltA) and five binding subunits (pentameric pltB). Of note, the toxin is only secreted when *S. Typhi* is intracellular and inside a *Salmonella*-containing vacuole (SCV) <sup>136</sup>.

The discovery of the typhoid toxin has added greater insight into the ability of *S. Typhi* and *S. Paratyphi A* to induce typhoid fever in humans <sup>137</sup>. Typhoid toxin has been shown in experimental animals to be important for orchestrating the clinical symptoms associated with acute typhoid <sup>138</sup>. Further evidence for the role of typhoid toxin during acute typhoid fever is the fact that both the *S. Typhi* and the *S. Paratyphi A* genome harbour almost identical copies of the toxin encoding genes <sup>139</sup>. Typhoid toxin has also been proposed to be a key factor for persistent asymptomatic infection <sup>140,141</sup>, a complex process in which the infecting pathogen can avoid clearance from the gallbladder. Furthermore, new research has revealed why *S. Typhi* and *S. Paratyphi A* do not cause typhoid fever in any other species. The precise binding of subunit *pltB* to Neu5Ac-terminated glycans exclusively expressed on human cells, which contrasts to the Neu5Gc-terminal glycan expressed on cells in other mammals, has added new insight into the host specificity of typhoidal *Salmonella* <sup>142</sup> (C57Bl/6 mice naturally expresses Neu5Ac <sup>142</sup>). Typhoid toxin targets not only the infected cells but also immune cells <sup>143</sup>. The toxin has been demonstrated to target neutrophils, lymphocytes, monocytes, and macrophages, which assists *S. Typhi* in manipulating the immune response, which may result in persisting an infection <sup>136,143,144</sup>. Of note, in mice injected with toxin circulating neutrophils were almost completely withdrawn <sup>136</sup>, contributing to obstructing neutrophil recruitment during typhoid acute phase <sup>143</sup>.

### **1.5.6 Pseudogenes**

Host-restricted pathogens have normally evolved from a broad host pathogen via horizontal acquisition of foreign genes or/and by inactivation of functional genes (reductive evolution) <sup>145,146</sup>. Genes that encode extraneous functions in a determined

niche accumulate mutations and eventually lose function. Additionally, to escalate fitness, genes encoding functions no longer harmonious with a specific lifestyle are selectively inactivated <sup>101,147</sup>. The *S. Typhi* genome contains approximately 5% pseudogenes in comparison to 1% in *S. Typhimurium* <sup>97</sup>, which are usually related to increased virulence in *S. Typhi* <sup>100</sup> or host-specificity <sup>148</sup>.

*Sif* and *Ssej* are two SPI-2 TTSS effectors required for the intracellular proliferation of *S. Typhimurium* <sup>149,150</sup>; *sif* has followed a differential evolutionary route in nontyphoidal *Salmonella* <sup>151</sup> and *ssej* is a pseudogene in *S. Typhi*. The loss of *ssej* in *S. Typhi* results in elevated cytotoxicity and reduced intracellular proliferation of *S. Typhi* inside cultured epithelial cells, which influences serovar adaptation during systemic infection <sup>152</sup>. *SopD2*, a known SPI-1 TTSS effector, which is expressed under SPI-1 inducing conditions in *S. Typhimurium*, is also a pseudogene in *S. Typhi*. The complementation of a functional *sopD2* gene into *S. Typhi* interferes with proliferation within human epithelial cells and decreases cellular permeability, leading to less cytotoxicity in the host cells <sup>153</sup>. Inactivation of *sopD2* is another example proposing that gene pseudogenization is vital for facilitating *S. Typhi* to cause systemic infection. Several other *S. Typhi* pseudogenes, such as *sopA* and *sopE2* <sup>100</sup>, *marT* <sup>101</sup>, *sptP* <sup>154</sup> all contribute to the serovar adaptation and aid systemic infection.

## **1.6 The pathogenesis of enteric fever**

*S. Typhi* and *S. Paratyphi A* begin to induce their infection in immunocompetent human's individual after ingestion. After crossing the intestinal mucosal barrier, they arrive at the lymphoid follicles, encountering non-phagocytic immune cells and

macrophages in which the bacteria have to survive and replicate (*S. Typhi* is a facultative intracellular pathogen of macrophages). The survival inside cells aids in the bacteria continuing its dissemination to distant sites<sup>99</sup>. To achieve successful systemic dissemination, *S. Typhi* and *S. Paratyphi A* have to possess specific strategies to invade particular cells and survive the insults of the highly sophisticated innate and adaptive immune systems. Our knowledge regarding the pathogenesis of enteric fever is largely derived from our understanding of *S. Typhimurium* in murine models. However, accumulative studies have shown that the behaviour of *S. Typhimurium* and *S. Typhi* are not comparable and *S. Typhimurium* virulence genes may not be as relevant in *S. Typhi*<sup>100,154</sup>. Recently, a humanized mice model has been reported to be capable of studying the pathogenesis of *S. Typhi*<sup>155–157</sup>. These models complemented immunocompromised mice, who were not able to generate antibody or T cells, with human induced pluripotent stem (iPS) cells. The human iPS cell engrafted mice were demonstrated to be susceptible to *S. Typhi* infection and even able to show the different immune responses when infected with wild type or mutant *S. Typhi*<sup>155–157</sup>. Another mouse model for studying typhoid is based using TLR-11 knockouts<sup>158</sup>. Intestinal TLR-11 in mice recognizes of *Salmonella* flagellin and *tlr11*<sup>-/-</sup> mice were found to be susceptible to *S. Typhi* when administered orally<sup>158</sup>. In the 1960s, the University of Maryland conducted human challenge model studies, which are acknowledged to be one of the key events that improved our understanding the pathogenesis of *S. Typhi*<sup>159–161</sup>. More recently, the Oxford Vaccine Group (OVG) has established another controlled human infection of wild type *S. Typhi*. These studies provided data on the correlation between the pre-challenge immunological status and the subsequent clinical outcome. Furthermore, this model delivered detailed kinetics and characteristics of innate as well as

adaptive immunological responses following infection with wild type *S. Typhi* <sup>162–169</sup>.

### **1.6.1 *Salmonella Typhi* survives the innate immune system**

#### **1.6.1.1 Observing facts indicating a distinct pathogenesis**

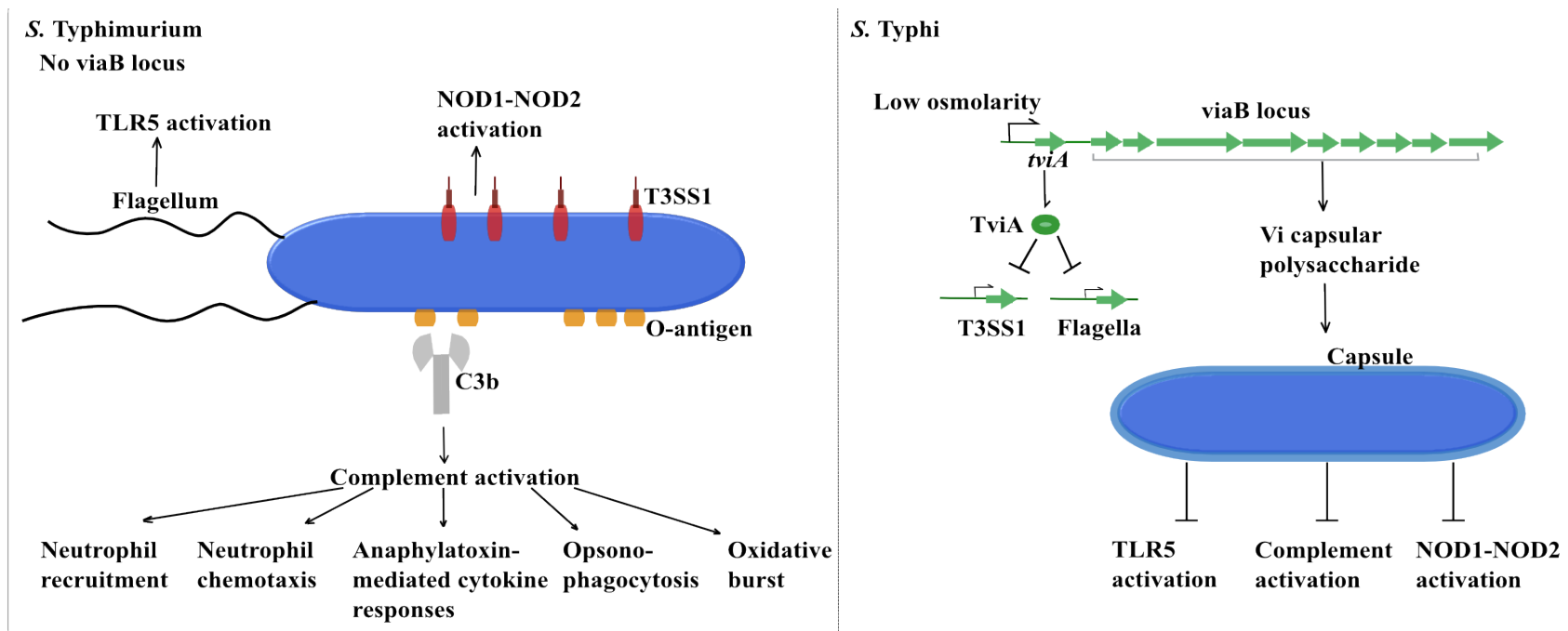
NTS induce a rapid onset of infection after 12-72 hours of incubation period; the typhoidal *Salmonella* serovars tend to initial enteric fever 8-14 days after entering a human body. While diarrhoea is considered the main symptom of NTS infections it occurs infrequently (33%) of the enteric fever patients <sup>14,170</sup>. Histopathology of biopsies of the upper small intestine from typhoid patients is typically dominated by mononuclear cell infiltrates and neutrophils are scarce <sup>171</sup>. Additionally, enteric fever has a long period of clinical symptoms and is a systemic infection while gastroenteritis has a short course of presentation and the infection is limited to the intestine and mesenteric lymph nodes <sup>14,170</sup>. Collectively, these different clinical manifestations represent distinct mechanisms of pathogenesis that assist the typhoidal *Salmonella* serovars in evading the innate immune defensive system, enabling them to cause systemic infections.

#### **1.6.1.2 *Salmonella Typhi* immune evasion**

On the process of *S. Typhi* invading epithelial cells and surviving the phagocytes, the bacteria senses the osmolarity changes of the approaching environments and alters its virulence expression through *tviA*, the first gene of *viaB* locus. *TviA* is induced to express at low osmolarity condition and positively regulates genes encoding the Vi capsular polysaccharide and negatively regulates genes encoding flagellin and T3SS-1 (Figure 1.1) <sup>129,130</sup>. Consequently, prior to entering the

intestinal mucosa (high osmolarity;  $\geq 0.3$  M NaCl) *S. Typhi* is non-capsulated, motile (flagellated) and invasive however it is capsulated, non-flagellated and does not express TTSS-1 when residing into the intestinal epithelial cells (low osmolarity;  $\sim 0.15$  M NaCl) <sup>118,130</sup>. These regulations of *tviA* minimize the alertness of the host immune defence resulting in neutrophil recruitment failure <sup>130</sup>. Neutrophils are one of the important factors the hosts possess to limit dissemination of *S. Typhimurium* infection in humans <sup>172</sup>. To perform their phagocytosis function, neutrophils must migrate to the sites of the detected threats. *S. Typhi* performs several ways to obstruct neutrophil migration.





**Figure 1.1** Immune evasion during typhoid fever (Redrawn and adapted from Keestra-Gounder, 2015<sup>173</sup>)

Comparison of mucosal responses elicited by *Salmonella* Typhimurium and Typhi. A) Injection of T3SS1 effector proteins by *S. Typhimurium* into intestinal epithelial cells activates the nucleotide-binding oligomerization domain containing 1 (NOD1)–NOD2 signalling pathway, thereby increasing neutrophil recruitment. The O-antigen chains of lipopolysaccharide (LPS) trigger the alternative pathway of complement cascade, which consequently promotes neutrophil chemotaxis, opsonophagocytosis and the generation of an oxidative burst. Anaphylatoxins produced during complement activation escalate cytokine production, which additionally accelerates recruitment of neutrophils into the threatened sites. *S. Typhimurium* flagellin stimulates Toll-like receptor 5 (TLR5), which activates CD4+T cell responses to the antigen in the intestinal mucosa. B) Induction of *tviA* expression by low osmolarity when the *S. Typhi* enters the intestinal mucosa, which induces to express Vi capsular polysaccharide and represses the expression of T3SS1 and flagella. Vi capsule obstructs the activation of complement by the *S. Typhi* O-antigen, which attenuates neutrophil recruitment, neutrophil chemotaxis, opsonophagocytosis and the ability of neutrophils to generate an oxidative burst. Repression of T3SS1 expression prevents the NOD1–NOD2 signalling pathway, which further reduces neutrophil recruitment. The impairment of flagellum expression blunts the development of CD4+T cell responses against flagellin. *S. Typhi* exploits these mechanisms to prevent activation of a number of pattern recognition receptors that assist the host responses against non-typhoidal *Salmonella* (NTS) serovars. C3b, complement component 3 fragment b<sup>173</sup>.

The physical outmost structure of *S. Typhi*, Vi, obstructs the generation of downstream signals that stimulate the host to recruit neutrophils to the site of infection. The structure of Vi prevents deposition of C3b (by the alternative pathway and limited to early time points after infection), which is the first complement deposition of the complement cascade activation <sup>173,174,175</sup> (Figure 1.1). By prevention of C3b deposition, *S. Typhi* is able to escape the opsonophagocytic activity by neutrophils <sup>175</sup>. The complement cascade is blocked, which leads to a lack of C5a deposition. Therefore, neutrophil chemotaxis does not occur, resulting in *S. Typhi* not being cleared by neutrophils. *S. Typhi*, unlike NTS serovars, does not have long O-antigen chains, which allows the Vi capsule to shield the LPS <sup>176</sup>. Further, the absence of C5a also lessens the recognition of the lipid A fragments of the pathogen LPS to TLR4, which reduces the cytokine response to provoke neutrophils recruitment to the site of infection <sup>177-179</sup>. A lack of interaction of C5a and its receptors inhibits neutrophils to generate an oxidative burst which is seen during *S. Typhimurium* infection <sup>173,180</sup>. Therefore, Vi permits *S. Typhi* to be virtually “invisible” to neutrophils <sup>181</sup>. However, given that *S. Paratyphi A* lacks Vi, there must be multiple alternative strategies for typhoidal *Salmonella* serovars to evade the host innate immune system <sup>182</sup>.

*S. Typhi* does not evoke the innate immune system as it blocks activation of TLR5 (absent flagella) and NOD1-2, and NLRC4 (absent TTSS-1 effectors) <sup>173,174</sup> (Figure 1.1). Down regulation of flagellin expression reduces TLR5-dependent responses in the intestinal epithelial cells <sup>183</sup>. This impairment contributes to an inhibition of inflammasome activation in macrophages in the early phase of infection <sup>119</sup>, this also

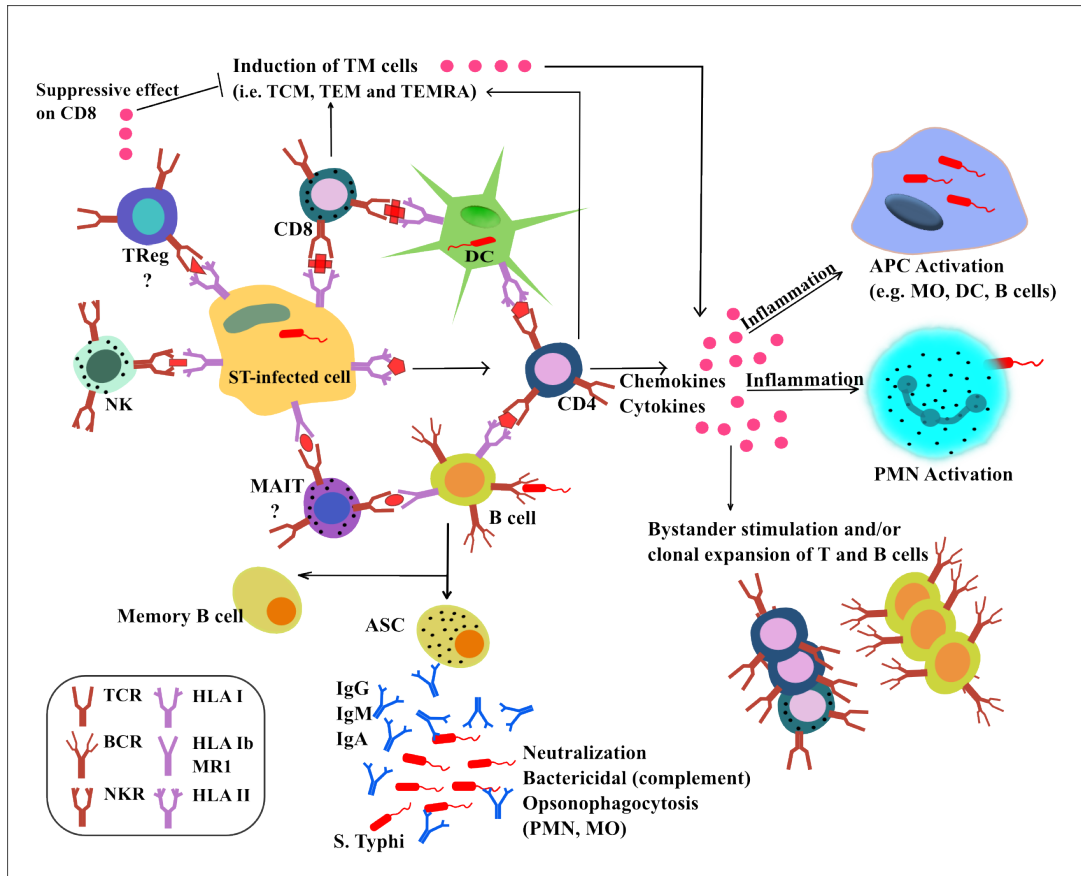
obstructs the maturation of CD4+ T cells against flagellin in the intestinal mucosa and the mesenteric lymph nodes <sup>184</sup>. *S. Typhimurium* pathogenesis experiments have shown that upon entry into epithelial cells, virulence effectors secreted by TTSS-1 activate NF- $\kappa$ B through the NOD1-2 signalling pathway, which leads to expression of IL8, an important cytokine that regulates inflammation in the gut <sup>185</sup> (Figure 1.1). Therefore, by rapidly suppressing TTSS-1 after successfully being internalized into intestinal epithelial cells, *S. Typhi* limits early inflammatory responses <sup>186</sup>. Alternatively, through suppression of TTSS-1, *S. Typhi* impairs the capacity of macrophages to detect pathogen-induced processes leading to a reduction in the release of intracellular bacteria through pyroptosis <sup>187</sup>. By reducing pyroptotic macrophage cell death, *S. Typhi* can persist in protected intracellular niches, while avoiding being up-taken and killed by neutrophils <sup>119</sup>.

Peyer's patches (PPs) are lymphoid follicles comprised of an arrangement of immune cells, such as B cell follicles, T cells, macrophages, dendritic cells, and other antigen-presenting cells. PPs play a crucial role in secretion of immunoglobulin A during *Salmonella* infection <sup>188</sup>. The whole patch is overlaid by microfold cells (M cells) which has been assumed to be the main entrance for *S. Typhi* during invasion <sup>188</sup>. *In vitro* *Salmonella* infection experiments have demonstrated that sidestepping the PPs dramatically reduces mucosal immune responses against *Salmonella* <sup>189,190</sup>. More recent research has revealed that although *S. Typhi* can adhere and invade M cells, the organisms preferentially invades enterocytes, which is facilitated by fimbriae operon *stg*

<sup>123,191</sup>. By avoiding the traditional route of *Salmonella* invasion (M cells), *S. Typhi* has an additional approach to prevent triggering the innate immune system.

### **1.6.2 Adaptive immunity to *Salmonella Typhi* infection**

Our current knowledge of the adaptive host immune response to enteric fever mostly originates from *S. Typhi* infections rather than from *S. Paratyphi A* infections. In fact, our understanding of the adaptive host immune responses to *S. Paratyphi A* has only been acquired through natural infection while *S. Typhi* has been studied in natural infection, experimental challenge (controlled human infection model) and vaccination <sup>192</sup>. Both *S. Typhi* and *S. Paratyphi A* are facultative intracellular bacteria <sup>193</sup>, therefore immunity against infection should involve both humoral immunity (antibody) and cell mediated immunity (CMI). Even though the precise contribution of each adaptive immune response aspect remains largely unknown, antibodies are predicted to be involved in host protection against extracellular bacteria, while CMI is relevant for the intracellular organisms <sup>192</sup> (Figure 1.2).



**Figure 1.2** Simplified diagram of immunity to *Salmonella Typhi* in humans (Redrawn and adapted from Marcelo B. Sztein, 2014 <sup>192</sup>)

Immunity to *S. Typhi* involves multiple factors including various antigen-presenting cells (e.g., macrophages, dendritic cells, B cells) and effector cells (e.g., various effector and regulatory T-cell subsets, B cells, NK, and MAIT cells). APC, antigen-presenting cells; ASC, antibody secreting cells; DC, dendritic cells; CD8, CD8+ T-cells; CD4, CD4+ T-cells; MAIT, mucosal associated invariant T-cells; MO, macrophages; NK, natural killer cells; PMN, polymorphonuclear neutrophil; TM, memory T-cells; TCM, central memory T-cells; TEM, effector memory T-cells; TEMRA, effector memory expressing CD45RA T-cells; Treg, regulatory T-cells; HLA, human leukocytes antigen; HLA-I, HLA class I; HLA-II, HLA class II; BCR, B cell receptor; TCR, T-cell receptor; MR1, HLA-I non-classical (b) molecule MR1; Ig, immunoglobulin.

### 1.6.2.1 Humoral immunity

There have been numerous studies on serum antibody responses to *S. Typhi* following natural infection, immunization, and human controlled infections recently. Several classes of antibodies such as IgG, IgM, and IgA (including SIgA in the intestinal fluid) against classical antigens such as O antigen, H antigen, Vi, and heat-shock proteins (GroEL, etc.), have been extensively examined in serum from acute and convalescent typhoid patients<sup>194–200</sup>. While there are studies showing a correlation between elevated anti *S. Typhi*-specific antibodies and mounting protective immune responses, susceptibility to *S. Typhi* infection has also been suggested within individuals with rising levels of *S. Typhi*-specific antibodies<sup>132,198,201,202</sup>. Therefore, the protective roles of antibodies to these antigens in protection against typhoid infection is conflicting and fragmentary<sup>192</sup>.

The role of anti-O-antibody in immune protection has been suggested from field studies of Ty21a vaccine, where raised anti-O-IgG was associated with protection when the vaccine is in the enteric-coated capsule formulation<sup>203</sup>. In the recent human controlled infection model, the concentration of pre-challenge anti-H and anti-Vi antibodies did not correlate with subsequent protection from development of typhoid<sup>204</sup>. In the same challenge study, IgG, IgM, and IgA against LPS and H antigens (but not Vi) were detected in the participants that developed typhoid. However, the Vi polysaccharide vaccine achieves comparable levels of protection to those of the oral live attenuated Ty21a vaccine<sup>205</sup>. Evidence for the efficacy of Vi polysaccharide vaccine suggests that high anti-Vi antibodies are protective against typhoid. Notably, for the Vi-rEPA

conjugate vaccine, a concentration of serum anti-Vi antibodies of 1.4–2.0 µg/ml has been documented to be protective <sup>206</sup>. Additionally, the majority of chronic *S. Typhi* carriers, especially in endemic areas, have high levels of serum anti Vi-IgG <sup>207</sup>. However, there have been very few reports on *S. Typhi* specific antibody function following *S. Typhi* infection.

In typhoid infection, B cells do not only serve as antibody secreting cells (ASCs), they also act as excellent antigen presenting cells (APCs) following the bacteria being internalized <sup>208–216</sup>. The serum *S. Typhi* specific ASCs peak at ~7–10 days after antigen encounter, before they home to the original mucosal sites for local antibody secretion <sup>159,217–223</sup>. The extended circulation of these particular cells in peripheral blood can be observed with prolonged exposure to these antigens <sup>224</sup>. Furthermore, the concentration of serum *S. Typhi* specific antibodies were observed to be elevated in correspondence with an increase in *S. Typhi* specific ASCs, which are thought to have been stimulated by antigen encounters at the mucosal surface; antibody titres to specific antigens were undetectable when the count of ASCs was low <sup>225</sup>.

#### **1.6.2.2 Cell mediated immunity (CMI)**

Cell mediated immunity (CMI), has been clinically observed to play an important role in the host defence against *Salmonella* infection <sup>192</sup>. Individuals that are deficient in IFN-γ, IL-12, and IL-23 are more susceptible to *Salmonella* infections, including *S. Typhi* and *S. Paratyphi A* <sup>226–228</sup>. Further, it has been demonstrated that in a Vietnamese population, variations within HLA-DRB1, HLA-DQB1 alleles and the tumour necrosis

factor TNF- $\alpha$  gene were significantly associated with being resistant or susceptible to typhoid fever <sup>229</sup>. Studies in *S. Typhi* and *S. Paratyphi A* infected patients in Nepal <sup>230</sup>, in Indonesia <sup>231</sup> and Israeli travellers to Nepal <sup>232</sup> found the serum pro-inflammatory (including IL-6, IL-8, IL-15, TNF- $\alpha$  and IFN- $\gamma$ ) and anti-inflammatory cytokines/mediators (including IL-10, TNF- $\alpha$  receptor (TNF-R) p55 and TNF-R p75) were significantly elevated during the acute phase of infection. Comparable patterns of pro-inflammatory cytokines in *S. Typhi* and *S. Paratyphi A* infections proposes that host immunity might be evoked by similar mechanisms in these patients <sup>192</sup>. Suppressive and pro-inflammatory responses always act in concert together to induce effective immune responses, which is thought to be vital for establishing a balanced immune response against *S. Typhi* infection <sup>192</sup>.

CMI response to *S. Typhi* infection involves largely CD<sup>4+</sup> and CD<sup>8+</sup> T cells. A range of pro-inflammatory cytokines including INF- $\gamma$  generated by *S. Typhi* specific CD<sup>4+</sup> and CD<sup>8+</sup> T cells have been reported following immunization <sup>233,214,234,235,212</sup>. However, immune response through CD<sup>4+</sup> or CD<sup>8+</sup> T cells is dependent on the triggering antigen <sup>192</sup>; *S. Typhi* soluble antigens appear to better at activating the response of CD<sup>4+</sup> T cells while *S. Typhi* infected cells are more likely to stimulate CD<sup>8+</sup> T cells <sup>209,212,214,236</sup>. The relationship between CMI and humoral immunity is not clear, and the correlation between those two types of adaptive immunity appear to vary from individual to individual following immunization with live attenuated *S. Typhi* <sup>212,237</sup>. This observation suggests that multiple immunological elements as well as the influence of host factors



(i.e. genetics, microbiome composition) determine the development and dominance of CMI and/or humoral immunity <sup>192</sup>.

### 1.6.3 Host specificity

*Salmonella enterica* serovars can commonly cause infections in a broad-range of vertebrate hosts including humans, while some serovars are host restricted, causing disease in a limited range of host species <sup>238</sup>. Only *S. Typhi* and *S. Paratyphi A* (and *S. Paratyphi B* and *C*) are adapted to be exclusive human pathogens. The current theory into this is that host specificity arises through two pathways, which contribute to the process by which *S. Typhi*/*S. Paratyphi A* are unable to thrive in other environments. Firstly, non-typhoidal *Salmonella* can effectively counter cell-intrinsic host defense mechanisms that restrict intracellular pathogen replication by targeting a Rab GTPase <sup>239</sup>. Rab GTPases, with more than 60 members, are global regulators of all steps of membrane budding in eukaryotic cells <sup>240,241</sup>. Rab32 is abundant in many different cell types and is required for killing *S. Typhi* in mouse macrophages <sup>242</sup>. Rab32 is recruited into the SCV and apparently delivers an antimicrobial agent (an unknown factor) to limit *Salmonella* replication. *S. Typhimurium* challenges this by secreting two TTSS effector proteins to block the host defensive Rab32-pathway in two converging mechanisms. One of the effector proteins (GgtE) is a protease, which can recognize and efficiently cleave Rab32 <sup>242,243</sup>. The other effector (SopD2) works as a GAP (GTPase-activating proteins, enzymes that alter post-translational modification) directly inhibiting Rab32-dependent pathway <sup>239</sup>. However, *S. Typhi*, not able to survive in mouse macrophages <sup>244</sup>, does not possess *ggtE*; *sopD2* is a pseudogene <sup>98,153</sup>. These

factors suggest that within the human host these two proteins are not under selective pressure to be maintained, otherwise this restriction pathway would not function in humans.

The loss of GgtE and SopD2 are not exclusively responsible for the host specificity of *S. Typhi*. Experimental evidence found that chimpanzee are permissive for *S. Typhi*, and replication of the infecting bacteria occurs at an equivalent level to those observed in humans<sup>245</sup>. However, ‘typical’ typhoid symptoms do not develop during *S. Typhi* infection in chimpanzees, suggesting that pathogen restriction does not solely determine host specificity of *S. Typhi*. Typhoid toxin is now considered to be central for classic acute symptoms of typhoid fever<sup>136</sup>. As described previously, the pentameric binding subunit PltB of typhoid toxin specifically binds to a receptor, which is predominantly expressed on human cells but not in other mammals<sup>142</sup>. The selective binding of PltB to its specific receptor expressed on the inside of the SCVs assists the toxin in being packaged into vesicle carriers which transport the toxin to the extracellular milieu<sup>246</sup>. After being trafficked to extracellular environment, typhoid toxin targets new cells expressing compatible receptors to intoxicate them. The target cells are neutrophils, lymphocytes, and even brain endothelial cells<sup>247,143</sup>. These two known evolutionary mechanisms, to prevent *S. Typhi* surviving in a non-permissive host and toxin binding to Neu5Ac-terminal glycans begin to explain the host specificity of *S. Typhi*.

## 1.7 Carriage

Chronic *S. Typhi* asymptomatic carriers have been documented for more than a century since the story of Typhoid Mary was reported. Mary Mallon was a New York cook who apparently transmitted typhoid to at least 51 people <sup>248</sup>. Approximately 2-5 per cent of the enteric fever cases in the typhoid endemic regions, are thought to become chronic carriers <sup>76,249</sup>. However, up to quarter of chronic carriers never experience an episode of acute enteric fever <sup>170</sup>. Chronic carriers are those who experience (or not) enteric fever and fail to clear the pathogens within one year of disease recovery <sup>250</sup>. The establishment of chronic carriage follows an acute infection and is associated with older age, being female, and having gallstones or an abnormality of the gallbladder <sup>170,250</sup>. Gallstones alone used to be considered the permissive niche for the bacteria to colonize and persist in the chronic stage <sup>251</sup>. *S. Typhi* may colonize the gallbladder and attach to gallstone surfaces during the acute phase of infection. The primary attachment to gallstones requires outer-membrane protein C (OmpC) followed by a specific interaction between flagellin and cholesterol <sup>120</sup>. Subsequently, the attaching bacteria form a biofilm surrounding the gallstones, the process is facilitated by the bacterial exopolysaccharide <sup>252</sup>. Free bacteria breaking from the biofilm matrix would enter the bile duct and access the small intestine before being shed in the faeces <sup>251</sup>. Invasion of gallbladder epithelium is an alternative mechanism by which *S. Typhi* establishes colonization in the gallbladder <sup>253</sup>. According to this hypothesis, intracellular *S. Typhi* replicate in epithelial cells and these cells follow a natural epithelial regeneration process. During this process, epithelial cells containing *S. Typhi* would be naturally expelled into the gallbladder lumen by the neighbouring cells <sup>185</sup>. Released bacteria

could invade new gallbladder epithelial cells or be washed back into the intestinal tract  
120,185.

The public health concern of *Salmonella* carriage is the shedding of viable bacteria, which causes new infection episodes in the community and facilitates disease transmission. Alternatively, individuals who have chronic irritation of their gallbladder because of persistent inflammation, abnormality or harbouring gallstones have been observed to be more likely to develop gallbladder carcinoma<sup>254</sup>. Furthermore, the carriage of *S. Typhi* significantly increases the risk of gallbladder carcinoma in these individuals<sup>255</sup>. More recent work has suggested that the biofilm formation plays a significant role in constantly secreting bacterial substances, which includes genotoxins and free radicals. These elements may subsequently induce DNA damage and mutations in gallbladder tissue<sup>256</sup>.

The prospective detection of chronic *Salmonella* carriers is a crucial step for preventing local enteric fever transmission<sup>257</sup> as well as beneficial to individuals at an awareness of the pathogens they are carrying<sup>254</sup>. However, the major challenge in identifying enteric chronic carriers is the fact that they are asymptomatic and may never have suffered from acute typhoid. Several attempts have been made to diagnose enteric carriers, which include sequential faecal culture<sup>258</sup>, bile/gallstone bacterial detection after cholecystectomy<sup>250</sup>, serological testing of raised anti-Vi antibody<sup>259</sup>, or the detection of metabolite markers of enteric chronic carriers<sup>257</sup>. However, for routine diagnosis, none of these methods are routinely applied<sup>257</sup>.

Various antimicrobial regimes have been applied to treat typhoid carriers yet none have been demonstrated to be wholly effective in eradication of pathogens <sup>64–66</sup>. Treatment failure might be associated with the biofilm formation, which prevents antimicrobial penetration or the development of pore-forming-bacterial subpopulations which are highly protected <sup>260</sup>. In the case of severe inflammation of the gallbladder, having a gallbladder removed (cholecystectomy) has a carriage cure rate of 85% <sup>261</sup>. However, persistence may still remain <sup>262</sup> because of the other chronic colonized foci in the biliary tree, mesenteric lymph nodes, liver, or bone marrow <sup>263</sup>.

## **1.8 The diagnosis of enteric fever**

The infected individuals and organisms associated with acute, convalescent, and asymptomatic carriage during enteric fever have different physiological characteristics <sup>264</sup>. There are various phases that an enteric fever diagnostic can be useful, these include clinical management (acute phase), disease tracking (convalescence and chronic carriage), and epidemiology (acute and convalescence) <sup>264</sup>. Therefore, the development of an enteric diagnostic should consider tests targeting different samples, exploiting different methods so that each test can be aimed at a specific purpose <sup>264</sup>. For the purpose of diagnosing acute enteric infections, the requirements include high specificity, sensitivity (as high as 100% as recommended by WHO <sup>265</sup>) low cost, and a short duration. Numerous novel approaches using leading-edge technologies, newly developed or tailored from other fields, tried to address these objectives such as detecting metabolites biomarkers of chronic typhoid carriage <sup>257</sup> and identifying

*Salmonella*-specific IgA responses in lymphocyte culture supernatant (TPTest) <sup>266,267</sup>.

Yet they are still restricted to research laboratories and are not promptly developed into simple, rapid diagnostic tests (RDTs) in most resource-limited laboratories <sup>264,265</sup>.

### **1.8.1 Blood culture and nucleic acid detection**

Microbiological culture is still considered as gold standard for enteric diagnosis because isolation of the causative agents from blood, bone marrow, rose spots or other sterile specimens is the most definite proof of enteric fever <sup>264</sup>. Bacterial isolation allows for subsequent whole-genome sequencing characterization of the circulating organisms and including their antimicrobial resistance genes <sup>265</sup>. Microbiological culture enables antimicrobial susceptibility testing on the isolated organisms, which can direct appropriate therapy <sup>264</sup>. Despite its absolute specificity, microbiological isolation (except from bone marrow) only has approximately 40 to 60% sensitivity <sup>268–271</sup>. Following infection trafficking, the typhoidal *Salmonella* serovars are presumably viable in various tissues, including blood. However, these pathogens may only be trafficked in blood for a short window, which is major factor limiting their detection in blood specimens <sup>265</sup>. Additional factors that limit the sensitivity of blood culture is that the quantity of the bacteria in blood, as low as <1 CFU/ml <sup>272</sup>, and many patients self-treat with antimicrobials prior to accessing a healthcare facility <sup>40,273</sup>. A further possibility is that *S. Typhi* is a fastidious pathogen, which is too delicate to survive the harsh environmental change from blood to culturing media <sup>265</sup>.

Nucleic acid amplification is largely considered to have most of the same difficulties for diagnosis as encountered with blood culture. *S. Typhi* and *S. Paratyphi A* PCR-based assays require only a small number of the bacteria in the clinical specimens and short time from laboratorial bench to results. The technique can also overcome the issue of dead or non-culturable bacteria as the result of antimicrobial pre-treatment. Multiple approaches of PCR-based assays ranging from conventional to real-time, monoplex to multiplex, have been developed to amplify the serovars' specific sequences including coding or putative genes <sup>264</sup>. The majority of the assays target the *fliC* gene, and have variable sensitivities and specificities <sup>274–281</sup>. Some of these methods claim the assays have 100% specificities and >90% sensitivity <sup>278,280,282</sup>, others report much more modest figures of 38 to 42% sensitivity <sup>281</sup>. In general, none of the PCR-based assays are used as a common enteric fever diagnostic tool across laboratories, which is evidence for the limited potential of these assays in the field <sup>265</sup>. The physical presence of the bacteria at low number in blood makes the required volume of blood even not feasible for a highly sensitive test. A novel method, which is termed “the selective target DNA enrichment method” (STEM), exploiting micrococcal nuclease added to ox bile to lyse human blood cells for subsequent enzymatic cleavage of the contaminating human DNA <sup>283</sup>. *S. Typhi* and *S. Paratyphi A* are not affected as they are bile-resistant and intact cells are collected by centrifugation and then subjected to PCR targeting *fliC-d* and *fliC-a* respectively <sup>283,284</sup>. The sensitivity of this approach was found to be 10<sup>3</sup> fold greater in comparison to non-pretreated PCR because human DNA is mostly eliminated <sup>283,284</sup>.

### 1.8.2 Serological diagnostic tests

The Widal test was developed in 1896 and was the first typhoid diagnostic. It is a visual test, which monitors agglutinating antibodies in blood of individuals with suspected enteric fever. The test targets the polysaccharide (LPS; O) and flagella (H) antigens of *S. Typhi* <sup>285</sup>. However, many of the surface antigens of *S. Typhi*, including O and H, are highly conserved with other members of the Enterobacteriaceae. The obvious problem of the specificity of the test is that people accumulate antibodies which are cross-reactive with *S. Typhi* as they age <sup>265</sup>.

ELISAs have also been applied to diagnose enteric fever and have again targeted Vi, liposaccharide (LPS; O), flagella (H) antigens and other outer-membrane proteins. It is understandable that the ELISAs detecting antibodies against O and H antigens have poor specificities and unimproved sensitivities in comparison to the Widal <sup>264</sup>. ELISA detecting antibody against Vi (anti-Vi IgG) may be a more suitable method for identifying chronic carriers <sup>259</sup> rather than those with acute infection (anti-Vi IgM) <sup>264</sup>.

Knowledge derived from developing serological assays for enteric fever has been applied to generate rapid diagnostic tests (RDTs). Currently available enteric fever RDTs are available in different formats and include immunodot, latex agglutination, dip stick, or lateral flow, which all detect IgM or IgG against *S. Typhi* antigens in blood (mostly) or other suitable specimens <sup>286</sup>. Overall, RDTs contribute some improvement over the Widal test for diagnosing enteric fever, yet still remain modest specificity and



sensitivity. Therefore, RDTs are not considered as a reliable approach for the diagnosis of enteric fever in endemic settings <sup>264</sup>.

## **1.9 Enteric fever vaccines**

Enteric fever occurs only in humans, making it a disease that can technically be eradicated <sup>182</sup>. Indeed, enteric fever has all but been eliminated from several countries in Southeast Asia where it was the most common cause of hospitalized febrile disease 20-30 years ago <sup>81,287</sup>. The elimination of typhoid in these areas is generally attributed to extensive improvements in sanitation rather than local immunization. However, for various political and economic reasons such improvements of national infrastructures cannot be promptly achieved in regions where enteric fever is currently endemic. Additionally, the treatment of enteric fever has encountered a major concern in the growing rates of AMR <sup>265</sup>. Collectively, such a situation makes it essential for the community to consider en masse immunization programmes targeting high-risk populations to minimize enteric fever where the disease is still common.

### **1.9.1 Inactivated whole cell typhoid vaccines**

The first typhoid vaccines introduced in the 1890s were the inactivated whole cell *S. Typhi* (heat-killed, phenol-preserved) which were parentally delivered <sup>288</sup>. In the 1960s, large-scale, randomized, controlled field trials implementing these vaccines was sponsored by the World Health Organization (WHO). These vaccines were found to be immunogenic with efficacies of 60 to 80%, thus conferring considerable protection against typhoid <sup>192,289</sup>. However, because of their excessive immunogenic reactions

(adverse fever and malaise) these vaccines were rarely used in high-risk populations in endemic regions and are no longer manufactured <sup>289</sup>.

### **1.9.2 First generation of live attenuated typhoid vaccines and Vi subunit typhoid vaccines**

Historically, the interest in developing typhoid vaccines faded soon after it was discovered that chloramphenicol treated enteric fever very effectively <sup>290</sup>. An efficacious typhoid vaccine generation attracted scientist's attention again in the 1970s, when chloramphenicol-resistant *S. Typhi* started to be isolated and caused several epidemics in Mexico and India <sup>49</sup>. The oral live attenuated *S. Typhi* vaccine strain Ty21a and parenteral purified Vi vaccine were licensed in the 1980s-1990s.

The oral live attenuated *S. Typhi* vaccine is comprised of the strain *S. Typhi* Ty21a, which was generated in the early 1970s by random chemical mutagenesis <sup>291,292</sup>. The key mutations that make Ty21a attenuated and safe for use in a vaccine are multiple mutations in galactose metabolism leading to *galE* deficiency <sup>292</sup>. Other spontaneous mutations attenuate the organism further include Vi, auxo-trophy for isoleucine and valine, and a mutation precluding H<sub>2</sub>S utilization <sup>291</sup>. Ty21a also has mutations in the *rpoS* gene, which was inherited from the wild-type parental strain Ty2 <sup>293</sup>. These mutations together limit the ability of Ty21a to survive starvation and different stress conditions. Ty21a was demonstrated to be only shed limitedly, which reduces the environmental risk <sup>294</sup>, a substantial benefit of a good oral vaccine. This oral live attenuated typhoid vaccine is licensed for children aged >6 years, providing 60-80%

efficacy, with 5-7 years lasting protection after three to four spaced doses <sup>295–297</sup>. Ty21a vaccine has also been shown to induce herd immunity <sup>296</sup>. Studies of immunological responses post immunization of Ty21a vaccine have dispensed much of our knowledge on the pathogenesis of *S. Typhi* <sup>298–301,301–303</sup>.

Parenteral subunit Vi polysaccharide vaccines were developed using non-denatured purified Vi capsular polysaccharide of *S. Typhi*. The Vi polysaccharide vaccine is administered as a single dose and confers protection of 55 to 72% in children aged >2 years for a period of 3 years <sup>304,305</sup>. The immunological induction of the vaccine stimulates neither mucosal immunity nor T cells because the vaccine is parenteral and Vi is a non-protein T-cell-independent antigen <sup>118</sup>. However, Vi vaccine has been demonstrated to induce herd immunity <sup>306</sup>.

### **1.9.3 Conjugate Vi typhoid vaccines**

The conjugation of the *S. Typhi* Vi polysaccharide and a protein carrier converts Vi, a T-cell-independent antigen, into a T-cell-dependent antigen. Therefore, conjugate Vi typhoid vaccines are able to induce the immune system to involve T helper cells, establishing immunological memory <sup>295</sup>. There are a number of candidates for this novel approach under development. One of the most well-known conjugates is the Vi O-Acetyl Pectin-rEPA conjugate vaccine of which the protein carrier is the non-toxic recombinant *Pseudomonas aeruginosa* exotoxin A (rEPA). This vaccine has been shown to be safe and immunogenic. A randomized controlled phase III field trial evaluated its efficacy of ~90% in children aged 2-5 years over 46 months of follow-up

<sup>134,170,206,307,308</sup>. Vi-conjugated to CRM197 <sup>309</sup> have been demonstrated to be safe and immunogenic in adults and teenagers <sup>310,311</sup>. Another conjugate has Vi linked to diphtheria toxoid <sup>312</sup> which has been licensed in India but peer review on its safety and immunogenicity has just been reported in a phase I trial in Filipino adults and children (aged 2-45 years) <sup>313</sup>. A relatively new Vi conjugate vaccine named Typbar-TCV™, developed by an Indian company (Bharat biotech), is a conjugate of Vi polysaccharide to tetanus toxoid, which is safe to administer to infants older than 6 months <sup>314</sup>. This vaccine has been licensed and is now WHO prequalified, the vaccine is currently being rolled out across South Asia and endemic parts of sub-Saharan Africa. The potential emergence of multi-drug resistant Vi negative *S. Typhi* variants may result in an increased incidence of typhoid fever caused by Vi negative *S. Typhi* if Vi subunit vaccines or conjugate Vi vaccines are generally implemented <sup>124,125</sup>. Consequently, Vi subunit vaccines or conjugate Vi vaccines will be no longer effective in the regions they are most expected to work <sup>192</sup>. New alternatives instead of the approach of using Vi as the main antigen stimulating the immune system have been considered. These include, among others, conjugates of *S. Typhi* and *S. Paratyphi A* LPS to carrier proteins or *Salmonella* proteins (e.g., flagellin, porins) to extend the generation of immunity to other relevant specific antigens.

#### **1.9.4 Novel attenuated live *Salmonella Typhi* strains**

Even though live attenuated Ty21a vaccine is well tolerated and efficacious, it requires at least 3 doses to achieve optimal immunogenicity. Engineering new attenuated strains that are as safe as Ty21a and immunogenic requiring a single dose is sought to improve

public health at regions where typhoid fever is endemic <sup>315</sup>. The aim of developing a new generation of attenuated strain candidates was to attenuate *S. Typhi* by mutating known virulence genes. There have been several candidate mutants generated (mostly using the same parent strain as Ty21a; Ty2) and some of them have been tested in humans. Many of the engineered organisms generated promising primary immunological data for further evaluation <sup>316</sup>. Strain M01ZH09 was a targeted mutant of *aroC* and structural protein *ssaV* in pathogenicity island 2 (SPI-2), which helps bacteria survive the intracellular oxidative burst. The mutated organism cannot efficiently spread out of the cell and cause systemic infection <sup>233,317</sup>. This strain was tested in humans and several clinical trials demonstrating a good safety profile and an ability to induce immune responses in children <sup>233,318,319</sup>. M01ZH09 strain was recently evaluated for protective efficacy after a single dose by direct challenge of susceptible volunteers <sup>168</sup>. Even though a single-dose of M01ZH09 did not succeed to provide substantial protection, there have been numerous of publications on the humoral and CMI responses to *S. Typhi* induced through this challenge <sup>168,220,233,318,319</sup>. Strain Ty800 was a *phO/phQ* virulence regulon mutant, which prevents the bacteria surviving inside macrophages <sup>315</sup>. There are also other candidates of which the targeted attenuations rely solely on mutating the synthesis of aromatic amino acids (*aroC* and *aroD*) or in combination with a mutation in heat-shock proteins (*htrA*). Strains CVD906 and CVD908 contain mutations in the two *aro* genes only and are generated from two different parental strains (ISP1820 and Ty2, respectively) <sup>320</sup>. Results from two phase I trials reported that CVD908 could induce appropriate immune responses <sup>320,321</sup> but also evoked a self-limiting vaccine bacteraemia 4-8 days after vaccination in 50% of

volunteers who received a low dose ( $5 \times 10^7$ ) and 100% of volunteers who received a high dose ( $5 \times 10^8$ ) <sup>321</sup>. The vaccine bacteraemia associated with CVD908 prevented it being tested in larger populations and efforts were sought to further attenuate the organism <sup>316</sup>. Strain CVD908-htrA was designed on from CVD908 and had an additional defined mutation in its heat-shock protein (htrA) <sup>217</sup>. This candidate has been evaluated in phase I and II clinical trials and was demonstrated to be safe and able to induce humoral and CMI responses <sup>221</sup>. However, none of the candidates described (strain M01ZH09, Ty800, CVD906, CVD908, CVD908-htrA) could stimulate serum anti-Vi antibody. Strain CVD909 was engineered from CVD908-htrA and harboured a strong promoter ( $P_{tac}$  instead of  $P_{tviA}$ ), which ensures the constitutive expression of Vi <sup>322</sup>. However, when volunteers were immunized with one or two doses of CVD909, serum anti-Vi IgG antibody was not detected despite anti-Vi antibody-secreting cells being identified in the majority of the volunteers <sup>219,322</sup>.

#### **1.9.4 Paratyphoid vaccines**

The first *S. Paratyphi* vaccines, produced over a century ago, were a component of the killed whole-cell parenteral typhoid vaccines <sup>299</sup>. The vaccines consisted of heat-inactivated and phenol-preserved *S. Typhi*, *S. Paratyphi* A, and *S. Paratyphi* B (TAB vaccine) <sup>299</sup>. However, their manufacture was discontinued because this vaccine was highly reactogenic <sup>323</sup>. Currently, there is no commercially available *S. Paratyphi* A vaccine. The current approach to *S. Paratyphi* A vaccine development follows two main strategies, which are O-specific polysaccharide (O:2) conjugates and live attenuated *S. Paratyphi* A strains. Monovalent *S. Paratyphi* A and bivalent *S. Paratyphi* A/*S. Typhi*

conjugate vaccines are under different stages of development <sup>323</sup>. The O:2 tetanus toxoid conjugate (O:2-TT) vaccine candidate, developed by the US National Institutes of Health (NIH), was demonstrated to be safe and immunogenic after the first dose at phase II trials, yet did not show an antibody boosting response after a second dose <sup>324</sup>. Another *S. Paratyphi A* vaccine candidate, developed by the GSK Vaccines Institute for Global Health, O:2 conjugated to CRM197. This conjugate was generated in an attempt to combine this with Vi-CRM197 in a bivalent formulation targeting both *S. Typhi* and *S. Paratyphi A*. The O:2-CRM197 conjugate has been shown to induce strong serum bactericidal activity against *S. Paratyphi A* and to be immunogenic when solely delivered or as a component of the bivalent formulation <sup>325</sup>. Another alternative conjugate candidate for immunizing against *S. Paratyphi A* is O:2-DT, yet the data from the clinical testing of this vaccine has not been released <sup>326</sup>. Regarding oral live attenuated vaccines against *S. Paratyphi A*, CVD1902 is a *S. Paratyphi A* strain harbouring two independently attenuating mutations in *guaBA* and *clpX* <sup>327</sup>. A single dose of this candidate was demonstrated, in phase I trial, to be well tolerated and immunogenic. A bivalent vaccine combining CVD1902 and CVD909 against both *S. Typhi* and *S. Paratyphi A* is the ultimate aim.

Lastly, there has been substantial attention in investigating live-vector vaccines using attenuated *S. Typhi* strains <sup>328</sup>. Multiple clinical trials have been conducted to examine the immunogenicity of the foreign antigens carried by an *S. Typhi* vector <sup>329–332</sup>. Even though the immune responses against the engineered *S. Typhi* foreign antigens are modest, current technologies promise great potential to improve the immunogenicity of

such vaccines <sup>328</sup>. This approach (using *S. Typhi* as vector) may also be an important avenue of development for a vaccine targeting both *S. Typhi* and *S. Paratyphi A* <sup>192</sup>.

### **1.10 Aims**

Enteric fever is a life-threatening disease causing major public health problems in LMICs. Further, the emergence of multi-drug resistant variants of *S. Typhi* and *S. Paratyphi A* in south Asia is making antimicrobial treatment for enteric fever progressively complicated. Potential control measures include improving the quality of drinking water supplies, education in better hygiene practices, better case detection, and widespread vaccination campaigns. Because the disease only occurs in humans and is faecal-orally transmitted, the former interventions are likely the most effective measures for enteric fever elimination. However, they cannot be promptly realized in the endemic areas. For example, Kathmandu (Nepal) and Chittagong (Bangladesh), have many of the typical issues in an endemic enteric fever region. Low quality infrastructure, including water supply and sanitary systems, appears to be ideal for the sustained transmission of these enteric pathogens, and are unlikely to be improved over the coming years. Therefore, improved diagnostic tests to assess disease burden and to subsequently manage the disease cases are a priority. In addition, mass immunization campaigns of safe, efficacious vaccines are another crucial arm for the rational control of enteric fever in these areas. Accordingly, a better understanding of the host immune responses against *S. Typhi* and *S. Paratyphi A* during infection, after infection and during chronic carriage is now essential. Alternatively, in a different setting in Vietnam, enteric fever was once a major problem in the 1990s, but the country has successfully controlled the



disease for the last decade or more. The model for controlling typhoid fever in Vietnam should be an exemplar for other settings where enteric fever is still a burden. However, the foundations leading to the elimination of the disease in this country has not been systemically reviewed and remains to be elucidated.

In order to address these issues, the aims of my thesis are:

1. To review the retrospective data and information regarding enteric fever in Vietnam and to combine this information with the country's economic development. The aim was to evaluate the greatest contribution for the decline and ultimate elimination of enteric fever in Vietnam in the last decade.
2. To measure the acute antibody responses against a range of recently identified *S. Typhi* protein antigens for the purpose of typhoid fever diagnosis in a cohort of Bangladeshi patients with febrile disease.
3. To assess the longitudinal dynamics of antibody against typhoid toxin in a cohort of enteric fever patients in Kathmandu, Nepal.
4. To identify immunogenic antigens that are targeted during bactericidal activity using a newly constructed *S. Typhi* transposon mutant library and assess the longitudinal dynamics of antibody against these identified antigens.

5. To estimate the prevalence of *S. Typhi* carriage in a typhoid endemic population (Kathmandu, Nepal) by exploiting their unique immunological profile.

## **Chapter 2 Materials and Methods**

### **2.1 Study sites and settings and ethics**

#### **2.1.1 Chittagong Medical College Hospital in Chittagong, Bangladesh**

Plasma samples used in Chapter 4 were collected at Chittagong Medical College Hospital (CMCH), Chittagong, Bangladesh. CMCH is a regional teaching hospital in the city of Chittagong, and co-located with the Chittagong Medical College, serving Chittagong and surrounding provinces <sup>333</sup>. The capacity of CMCH is of 1,000 inpatient beds admitting 600,000 patients (500,000 adults and 100,000 children) annually and sees > 5,000 outpatients daily. Chittagong is the second largest city in Bangladesh, with a of 2.1 million. It is positioned in South-eastern Bangladesh and has a tropical wet climate which has cool and dry winters from December and February <sup>334</sup>.

The study conducted at CMCH followed the principles expressed in the Declaration of Helsinki. The Bangladesh National Research Ethical Committee (BMRC/NREC/2010-2013/1543), the Chittagong Medical College Hospital Ethical Committee, the Oxford Tropical Research Ethics Committee (OXTREC 53-09) gave ethical approval for the study. Informed written or thumbprint consent was taken from the subject, their parent or caretaker for all enrolees.

Patients participated in this study was recruited by Prof Chrisopher Parry and plasma seperation was performed by the nurses of CMCH, Chittagong, Bangladesh.

### **2.1.2 Patan Hospital in Kathmandu, Nepal**

The majority of plasma samples in my study, which are used in Chapters 5, 6, and 7 originated from enteric fever studies conducted in Patan Hospital in Kathmandu, Nepal. The majority of patients are from the Kathmandu Valley and Lalitpur area. Patan Hospital has 318 beds with the occupancy rate about 85% admitting 16,000 inpatients annually. Kathmandu is the capital city of Nepal having a population of 1.5 million. The city is located at an altitude of 1,300m having four typical seasons of which the hot wet season is from June to August and is reported to have the greatest burden of enteric infections.

The plasma samples used in my study were collected from a clinical trial that received ethical approval from the Nepal Health Research Council, Kathmandu, Nepal and the Oxford Tropical Research Ethics Committee, Oxford, UK. The study was conducted according to the principles expressed in the Declaration of Helsinki. The trial was registered as ISRCTN63006567 ([www.controlled-trials.com](http://www.controlled-trials.com)). Written informed consent was taken from the subject, their parent or guardian of enrollee under 18 years of age.

The patient recruitment data, blood cultures, bile cultures, and plasma samples utilised in my work were from enteric fever studies conducted at Patan Hospital, Kathmandu, Nepal. The work providing these samples was performed by doctors, nurses and microbiologists based at this hospital.

## **2.2 Bacterial identification and plasma preparation**

### **2.2.1 Blood culture**

Enteric infections were definitively diagnosed by blood culture. Blood (1-2mL for children; 5-12mL for adults) was taken within 24 hours of admission prior to antimicrobial therapy was started in hospital. For blood culture in CMCH, blood samples were inoculated into an adult or paediatric BactAlert® blood culture bottle and incubated aerobically in a BactAlert® automated system (bioMérieux, Marcy l'Etoile, France) at 37°C for 5 days. For blood culture in Patan Hospital, blood samples were inoculated into a sterile bottle containing tryptone soya broth and sodium polyanethol sulfonate, up to a total volume of 50mL. The broth of bottles that were positive was subcultured onto blood, chocolate and MacConkey agar. Suspected *Salmonella* colonies were identified using standard biochemical tests and serotype-specific antisera (Murex Biotech, Dartford, England).

### **2.2.2 Bile culture**

Bile collected from all patients undergoing cholecystectomy or laparotomy surgery between June 2007 and October 2010 was submitted for culture. Selenite F broth and Peptone broth individually were prepared as an equal volume as the bile sample and made the bile inoculated mixture. The broth was incubated at 37°C overnight, then subcultured onto MacConkey agar and Xylose Lysine Deoxycholate (XLD) agar incubated at 37°C overnight. Cultured Gram-negative bacteria were identified by standard microbiological methods and API20E (BioMerieux, Inc). Potential *S. Typhi*

and *S. Paratyphi* A isolates were confirmed by slide agglutination by specific antisera (Murex Biotech, Biotech, England).

### **2.2.3 Plasma preparation**

Blood samples were collected in EDTA K3 (Golden Vac) containing 10% EDTA.

Plasma and pellet were separated by centrifuging at 3,000rpm for 3 minutes and plasma aliquoted into cryovials and stored at -20°C.

## **2.3 Plasma samples and bacterial isolates**

### **2.3.1 Plasma samples for Chapter 4**

Plasma samples used in Chapter 4 originated from a study conducted in Chittagong Medical College Hospital, Chittagong, Bangladesh <sup>335</sup> aiming to compare the accuracy of three rapid diagnostic tests for typhoid fever; the Life Assay Test-it™ Typhoid IgM (Life Assay Diagnostics (Pty), Cape Town, South Africa), the SD Bioline test (Standard Diagnostics Inc., Gyeonggi, Korea), and the CTK Biotech Onsite Typhoid IgG/IgM Combo Rapid-test cassette (CTK Biotech, Inc., San Diego, CA, USA). Briefly, adults and children (>6 months) consecutively admitted from January to June 2012 to the adult and paediatric wards at CMCH with an axillary temperature of  $\geq 38^{\circ}\text{C}$  up to 48 h after admission and history of fever for <2 weeks were eligible for the inclusion in the study. The plasma collection for this was from patients with confirmed typhoid fever (n=32, 16 cases confirmed by blood culture, 13 cases confirmed by PCR and 3 cases confirmed by both blood culture and PCR), other confirmed infections (n=17) and undiagnosed febrile

disease (n=244). Further, there were 40 plasma samples collected from among the hospital staff as negative controls.

### **2.3.2 Plasma samples for Chapter 5, 6, 7**

#### **2.3.2.1 Plasma samples for investigations of acute and convalescent febrile diseases**

Plasma samples used for longitudinal assessment originated from an open-label, randomized, controlled trial, comparing Gatifloxacin and Ofloxacin for the treatment of uncomplicated enteric fever, conducted at Patan Hospital. The trial was registered as ISRCTN63006567 ([www.controlled-trials.com](http://www.controlled-trials.com)). Briefly, patients were randomly assigned to treatment for 7 days with either ofloxacin (200mg or 400mg tablets, National Healthcare Pvt. Ltd., Nepal) at 20mg per kg per day in two divided oral doses or gatifloxacin (400mg tablets, Square Pharmaceutical Limited, Bangladesh) at 10mg per kg once daily. Blood from all patients were sampled on the day of enrolment and day 8, 28 (month 1) and 90 (month 3) post enrolment. Blood samples were directly subjected to culture or to plasma preparation. Number of plasma samples collected at day 1, day 8, month 1, month 3 from *S. Typhi* confirmed patients were 152, 148, 148, 81; from *S. Paratyphi A* confirmed patients were 68, 67, 66, 52; from unidentified febrile patients were 316, 316, 266, 177, respectively.

#### **2.3.2.2 Plasma samples for investigations of asymptomatic chronic enteric fever**

Patients admitted to the surgical department of Patan Hospital for open cholecystectomy or laparotomy surgery for symptomatic cholelithiasis, between June 2007 to October 2010, were enrolled after giving written informed consent. Blood samples were

collected and plasma samples were prepared as described in section 2.2.3. Forty-six blood samples including 10 from patients having confirmed *S. Typhi*, 6 from patients having confirmed *S. Paratyphi A*, and 30 from patients that were bile culture negative (cholecystectomy controls) were used.

#### **2.3.2.3 Plasma bank**

The plasma bank was collected from patients visiting Patan Hospital Emergency Department from January 2010 to September 2010. The bank consisted of 733 plasma samples collected from an age group spanning from 2 months to 65 years of age. The distribution of the plasma bank was  $28 \pm 2$  samples for each year of age from 0 (2 months) to 30 years;  $9 \pm 1$  samples for each year of age from 31 to 60 years old; and finally,  $4 \pm 1$  samples for each year of age from 61 to 65 years old. Community population was collected from 49 afebrile adults living within Kathmandu valley.

#### **2.3.3 Bacterial isolates**

The bacterial isolates used for genetic manipulation in Chapter 6 were *S. Typhi* AS252, identified in Ho Chi Minh city, Vietnam in 1999, and *S. Paratyphi A* 01TY546, identified in Kathmandu, Nepal in 2010.

### **2.4 Protein purification**

I selected 18 *S. Typhi* antigens that gave a differential serodiagnostic signal using protein microarray screening<sup>336</sup> for further expression and purification (Table 2.1). The coding sequences of the selected genes, excluding trans-membrane domains were PCR



amplified from CT18 genomic DNA and cloned into the 5'NcoI and 3'NotI restriction sites of pET28b(p) vector (Novagen, UK) for further His-Tag purification. *E. coli* DH5 $\alpha$  were transformed with the plasmid constructs for stable storage and *E. coli* BL21(DE3)pLysS (Promega, WI, USA) were used for expression and purification (Table 2.1)

**Table 2.1** Plasmid constructs of *Salmonella* Typhi antigens generated for protein expression

Target (CT18)	Construct name	5'RE	Ty2 nomenclature and Coding sequence	3'RE	Forward primer	Reverse primer	bp	aa	kDa
STY4190	pEK90	NcoI	3904 (Typhi) aa25-495	NotI	catg ccatgg gt GATGCGCTCCAGCCCGATC	gcatgagc gcggccgc CTGTGCCGCCGGTGTTC	1413	471	52.6
STY3208	pEK91	NcoI	2970 (Typhi) aa1-279	NotI	catg ccatgg gt ATGGCAGCTAACGGAGAAAATAATCC	gcatgagc gcggccgc CCAGGTCTTACCTATTTTAAATTCCC	837	279	30.5
STY1767	pEK92	NcoI	1224 (Typhi) aa21-154	NotI	catg ccatgg gt GCACCGGCGCCAAATGCCAG	gcatgagc gcggccgc AATTCGCCGTGCCTGCCAGAAG	402	134	15.1
STY1703	pEK93	NcoI	1285 (Typhi) aa1-124	NotI	catg ccatgg gt ATGCGTATTACCAAAGTTGAGGG	gcatgagc gcggccgc TTCGCTATTCTTAACATAGAATATCTC	372	124	14.1
STY1522	pEK94	NcoI	1459 (Typhi) aa25-363	NotI	catg ccatgg gt TGCACAACCCTTGCTATTTCAGGATAAAC	gcatgagc gcggccgc TCCTTTGACGTTGATTTTCTCGAACAC	1017	339	37.6
STY1886	pEK95	NcoI	1111 (Typhi) aa28-269	NotI	catg ccatgg gt AAAGTTATGACCTGGAATCTTCAGGG	gcatgagc gcggccgc ACAGCTTCGTGCCAAAAAGGCTAC	726	242	26.4
STY1498	pEK96	NcoI	1477 (Typhi) aa203-305	NotI	catg ccatgg gt GGCGTGATTGAAGGGAATTGATTCc	gcatgagc gcggccgc GACGTCAGGAACCTCGAAAAGCG	309	103	11.5
STY3375	pEK99	NcoI	3116 (Typhi) aa1-118	NotI	catg ccatgg gt ATGGCGTCCACATATCGCAC	gcatgagc gcggccgc CTCTTGTTGGATCGACTGGC	354	118	13.8
STY1372	pEK100	NcoI	1594 (Typhi) aa27-74	NotI	catg ccatgg gt AGCAACCGCGCCGGTCGG	gcatgagc gcggccgc GCGCTCTCTCCAGTTCGGATG	144	48	5.7
STY1612	pEK101	NcoI	1376 (Typhi) aa30-108	NotI	catg ccatgg gt AGTAAACAGAAAGAACGCCAGGC	gcatgagc gcggccgc TTGACCTCCGGTATTGCGGTAC	237	79	9.2
STY0357	pEK102	NcoI	2538 (Typhi) aa20-246	NotI	catg ccatgg gt GGTTTGCTGAGCAGCAGCAGC	gcatgagc gcggccgc TTTTGCCTCGGAGAGCGTATAATTTG	681	227	25.8
STY4539	pEK103	NcoI	4239 (Typhi) aa20-414	NotI	catg ccatgg gt CAGCAGACCTCCACCCAAACC	gcatgagc gcggccgc CGACCGGTCAGCCGGTTTATC	1185	395	41.9
STY0452	pEK104	NcoI	2449 (Typhi) aa22-179	NotI	catg ccatgg gt CCGCAGAGCGAAGTTCGC	gcatgagc gcggccgc TTGATTAACAGGCTGAATATCATGG	474	158	17.2
STY0796	pEK105	NcoI	2126 (Typhi) aa27-262	NotI	catg ccatgg gt CAGGCGCCAATCAGTAGTGTC	gcatgagc gcggccgc CATCGCGTTAAGACGCTTCTGC	708	236	25.4
STY0065	pEK106	NcoI	0058 (Typhi) aa33-79	NotI	catg ccatgg gt GGTATGTCTGCCGTGATTACTC	gcatgagc gcggccgc AGCGTTAAGCGGTGATGGTG	138	46	4.9
STY3093	pEK107	NcoI	2864 (Typhi) aa1-118	NotI	catg ccatgg gt ATGTTTTTGACGTACATTTTCagg	gcatgagc gcggccgc CTGGCCTTTGGCGTTAATTTTAC	354	118	13.6
STY1086	pEK108	PciI	1855 (Typhi) aa20-178	NotI	catg acatgt ta GGAGAAAATAAAAGCTATTATCAGCTC	gcatgagc gcggccgc AGCGGCCGCTTCCTGACTC	477	159	17.4
STY3765	pEK109	NcoI	3515 (Typhi) aa1-518	NotI	catg ccatgg gt ATGAAAGTAAACTGCTTGCTGCC	gcatgagc gcggccgc CTTCTTCACATCCGCAACACG	1554	518	55.5

For protein expression, *E. coli* BL21(DE3)pLysS harbouring unique plasmid constructs (pEK90 - pEK109) containing the genes of interest were inoculated into LB broth containing 100mg/L kanamycin (Sigma, MO, USA), and incubated at 37°C overnight. Overnight cultures were diluted (1:100) into LB broth (100mg/L kanamycin) and incubated at 37°C with agitation until optical density (OD at 600nm) of 0.5. Expression of the exogenous proteins was induced by the addition of isopropyl-b-D-thiogalactoside (IPTG) (Sigma Aldrich, UK), to a final concentration of 0.1mM. Bacterial cells were harvested by centrifuging (5000xg at 4°C for 10 minutes) after 3 hours of incubation at 24°C.

For soluble proteins, bacterial pellets were resuspended in 50mM phosphate buffer (pH8) containing 300mM NaCl and 10mM imidazole. After sonication, cell debris and the membrane fragment were pelleted by centrifugation at 16,000xg at 4°C for 30 minutes. Supernatants were filtered through a 0.45µm membrane before being rocked at 4°C with nickel coated agarose beads (Ni-NTA, Invitrogen) for 2 hours. Protein bound Ni-NTA beads were loaded into gravity flow columns (Qiagen, Germany) and washed with 20 mM imidazole in phosphate buffer. Proteins were eluted with 250mM imidazole in phosphate buffer. For insoluble proteins a denaturing protocol was performed by firstly incubating the bacterial cells in an 8M urea (pH7.8) solution containing 20mM sodium phosphate and 500mM NaCl. Proteins were eluted with 4M Urea (pH3) in a solution containing 20mM Sodium Phosphate buffer and 500mM NaCl. Proteins were renatured after purification in 50mM Sodium Phosphate solution and 500mM NaCl.

## **2.5 Enzyme-linked immunosorbent assays (ELISA)**

ELISAs to detect antigen specific antibodies, classes IgM or IgG, in human plasma samples were performed as described previously<sup>337,338</sup>. Briefly, 96 well flat-bottom ELISA plates (Nunc 2404, Thermo Scientific) were coated overnight with 100mL per well of the various antigens (final concentrations; 7µg/mL of protein antigens, 15µg/mL for O2 antigen, 1µg/mL for the STY1498, STY1479, Vi and typhoid toxoid antigen in 50mM Carbonate Bicarbonate buffer). Coated plates were washed and blocked with 5% milk solution in PBS. After 2 hours of blocking, plates were washed and incubated with 100mL (per well) of a 1:200 dilution of plasma at ambient temperature for 2 hours. Plates were washed again and incubated with 100mL per well of alkaline phosphatase-conjugated anti-human IgM at ambient temperature for 1 hour. The final ELISA plates were developed using p-Nitro- phenyl phosphate (SigmaFAST N1891, Sigma Aldrich, UK) substrate for 30 minutes at ambient temperature and the final absorbance was read at dual wavelengths (405nm and 490nm) using an automated microplate reader (Biorad). End point positive absorbance results were defined as optical densities (OD) greater than the absorbance obtained for the blank control wells plus four times the standard deviation. Three wells of culture *S. Typhi* positive plasma were run as control for every 96-well plate for each antigen. The results of each ELISA plate were accepted only if the OD values of the controls were within the range of their known values plus/minus two standard deviations of the blank wells. Every sample (plasma or positive control or blank control) was measured in triplicate (in three individual wells).

### **2.5.1 ELISAs specific for Chapter 4**

ELISAs to detect antibodies (IgM) against purified antigens and anti Vi polysaccharide (provided by Novartis vaccines for Global Health, Siena, Italy which were purified as described elsewhere <sup>309,325</sup>) in human plasma were performed as described in section 2.5. Of the 18 protein antigens I aimed to purify, I was initially able to express and purify 12 (Table 2.2). All these 12 proteins were used as the antigens for the ELISA performed in Chapter 4. I was additionally able to purify protein Hyle (STY1498) for later experiments described in Chapter 6.

For the purpose of the experiments in Chapter 4, I used plasma from patients with confirmed enteric fever (n=32), other confirmed infections (n=17), and afebrile controls (n=40). ELISAs were performed on plasma from 243 patients with undiagnosed febrile disease using the most specific antigens.

**Table 2.2** *Salmonella* Typhi antigens expressed for serological testing in Chapter 4

Gene ID Number <sup>a</sup>	Isotype detected using array <sup>b</sup>	Gene name	Amino acid identity <i>S. Typhi</i> CT18/ <i>S. Paratyphi</i> A AKU_12601	Annotation
STY0452	IgM	<i>yajI</i>	163/165 (98%)	Putative lipoprotein- Prokaryotic homologue of protein DJ-1
STY0796	IgM	<i>ybgF</i>	260/262 (99%)	Putative exported protein - Tol/pal system protein
STY1086	IgM	-	187/187 (100%)	Putative lipoprotein
STY1372	IgM	<i>pspB</i>	74/74 (100%)	Phage shock protein B
STY1612	IgM	-	Absent	Putative membrane protein - prophage associated
STY4539	IgM	<i>pilL</i>	Absent	Putative exported protein - type IV pili
STY1522	IgG	-	292/296 (99%)	Putative secreted protein - Choloylglycine secreted homologue
STY1703	IgG	<i>ssaP</i>	123/124 (99%)	Putative secreted protein - T3SS
STY1767	IgG	<i>nlpC</i>	48/140 (34%)	Putative lipoprotein - Endopeptidase
STY1886	IgG	<i>cdtB</i>	268/269 (99%)	Cytolethal distending toxin subunit B homologue
STY3208	IgG	-	277/279 (99%)	Hypothetical protein - Unknown function
STY4190	IgG	<i>yhjJ</i>	190/192 (99.0%)	Putative Zinc-protease

a) Reference Parkhill et al. 2001 <sup>339</sup>

b) Reference Liang et al. 2013 <sup>340</sup>

### 2.5.2 ELISAs specific for Chapter 5

ELISAs to detect IgM and IgG in human plasma samples against various antigens (Vi, O2, or typhoid toxin) were performed as described as section 2.5. The Vi and O2 antigens were provided by Novartis vaccines for Global Health (Siena, Italy), which were purified as described previously<sup>309,325</sup>. Typhoid toxin was provided by Professor Jorge Galan (Department of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT, USA), which was generated by mutating its catalytic subunits PltA<sup>E133A</sup> and CdtB<sup>H160Q</sup>. The overexpression and purification of typhoid toxoid were as described previously<sup>136</sup>.

For the purpose of experiments in Chapter 5, a collection of plasma samples from 220 enrolees with culture confirmed enteric fever (152 with *S. Typhi* infection and 68 with *S. Paratyphi A* infection) longitudinally sampled for up to 90 days was analysed. Additionally plasma samples from 316 febrile patients with clinical symptoms of typhoid but were blood culture negative, 49 plasma samples from healthy afebrile individuals living in the same population, and 733 plasma samples collected from the plasma bank were used for measuring background antibody concentrations in the population<sup>337</sup>. The plasma samples and tests performed in Chapter 5 are summarised in Table 2.3.

**Table 2.3** Plasma samples and ELISA tests detecting IgM and IgG performed in Chapter 5

<b>Samples</b>	<b>Anti-toxoid IgM</b>	<b>Anti-toxoid IgG</b>	<b>Anti Vi IgG &amp; anti O2 IgG</b>
<i>S. Typhi</i> confirmed (Day 1, Day 8, Month 1, Month 3)	152, 148, 148, 81	152, 148, 148, 81	0
<i>S. Paratyphi A</i> confirmed (Day 1, Day 8, Month 1, Month 3)	68, 67, 66, 52	68, 67, 66, 52	0
Unidentified febrile (Day 1, Day 8, Month 1, Month 3)	316, 316, 266, 177	316, 316, 266, 177	0
Afebrile community Control (AF)	49	49	
Serum bank (SB)	0	733	733



### **2.5.3 ELISA specific for Chapter 6**

ELISAs to detect IgG in human plasma samples against various antigens (STY0796, STY1086, STY1372, STY1498, O2, Vi) were performed as described as in section 2.5 using the same set of human plasma samples as used in Chapter 5 (section 2.5.2, Table 2.3).

### **2.5.4 ELISAs specific for Chapter 7**

ELISAs to detect specific IgG against antigens listed in Table 2.4 were performed as described in section 2.5. A collection of plasma samples collected from 148 patients with culture confirmed acute typhoid fever on day 8 after admission and from patients undergoing cholecystectomy which included 10 culture confirmed *S. Typhi* carriers and 30 cholecystectomy controls were used in this chapter. Additionally, 733 plasma samples from the plasma bank were used for measuring background antibody concentrations in the population<sup>337</sup>. The plasma samples and tests performed in Chapter 7 are summarised in Table 2.4.

**Table 2.4** Plasma samples and ELISAs detecting antibody IgG performed in Chapter 7

<b>Samples</b>	<b>Anti - STY1479</b>	<b>Anti- toxoid</b>	<b>Other antigens*</b>	<b>Anti-Vi</b>
<i>S. Typhi</i> carriage	10	10	10	10
Cholecystectomy control	30	30	30	30
<i>S. Typhi</i> acute (Day 8)	148	148	148	148
Serum bank (SB) year 1-19	351	351	0	351
Serum bank (SB) year 20-65	382	382	0	382

\*Other antigens included STY0452, STY0796, STY1086, STY1372, STY1498, STY1522, STY1612, STY1703, STY1767, STY3208, STY4190, STY4539

## **2.6 Typhoid toxin neutralization**

Plasma samples at all four different time points of four patients in every group of confirmed *S. Typhi*, confirmed *S. Paratyphi A* and undiagnosed febrile disease were selected randomly with respect to their seroconversion status. Eight community controls were also selected for the neutralization assay. Toxin neutralization assays were performed by examining the ability of the different plasma samples to block the DNA damage response induced by typhoid toxin in cultured epithelial cells<sup>341</sup>. Briefly, human Henle-407 epithelial cells were seeded onto 12-well plates at a concentration of  $2.5 \times 10^4$  cells per well and incubated at 37°C overnight in a tissue culture incubator. Purified typhoid toxin (6nM in 20µl of PBS) was incubated with 1µl of the different plasma samples for 30 minutes at room temperature and applied to the cultured cells, and further incubated for 68 hours at 37°C CO<sub>2</sub> in tissue culture incubator. Cells, were then washed, trypsinized, and processed for cell cycle analysis as previously described<sup>136</sup>.

The toxin neutralization experiments were performed in the laboratory of Professor Jorge Galan, Yale University School of Medicine, New Haven, CT, USA.

## **2.7 *S. Typhi* transposon mutant library preparation**

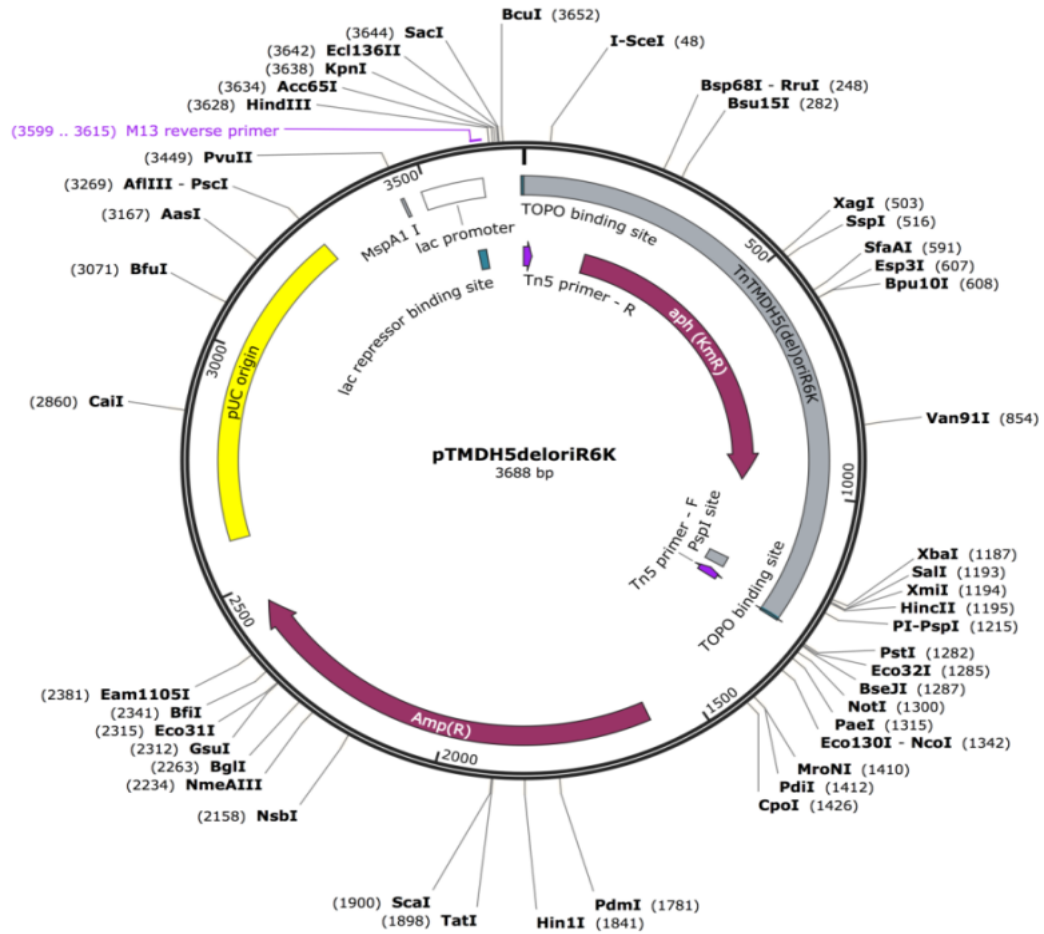
### **2.7.1 Competent cell preparation**

The *S. Typhi* transposon mutant library was generated with a clinical *S. Typhi* isolate AS252. Therefore, to get the starting materials for the library preparation, *S. Typhi* isolates AS252 was subjected to competent cell preparation. Pre-warmed LB broth was inoculated with a suitable volume of overnight AS252 bacterial culture at a proportion

of 100:1 and horizontally agitated at 37°C, ~180rpm, until early exponential phase (OD at 600nm ~ 0.3 - 0.4). The bacterial culture was cooled on ice and harvested by centrifuged at 4°C at 4,000rpm for 10 minutes using a temperature-controlled centrifuge (Eppendorf, Hamburg, Germany). The supernatant was discarded and the cells were re-suspended in a half of the initial volume of chilled (0 – 8°C) 10% glycerol. This procedure for harvesting and re-suspending cells was repeated twice with an increasing centrifugation speed (9,000rpm) before cells being re-suspended in 0.5mL of (0 – 8°C) 10% glycerol and centrifuged at 6,000rpm for 1 minute. The supernatants were discarded and the cells were suspended in  $\frac{1}{1000}$  x volume of 10% glycerol (i.e., if starting culture was 400mL then re-suspended in 400μl). The competent cells were kept on ice if to be used immediately or stored at -80°C for later use; 60μl of cell suspension was used for one electro-transformation. This procedure was also used for competent cell preparation in section 2.9.3.

### **2.7.2 High frequency electrotransformation of *Salmonella* Typhi**

The Tn5 transposon was constructed from the EZ-Tn5 < R6Kgori/KAN-2> (Epicentre Biotechnologies) flanked with T7 and SP6 promoters at each end, respectively, and with R6Kgori deleted <sup>342</sup>. The plasmid was named in house as pTnTMDH5(del)oriR6K (Figure 2.1). Other materials comprised of Phosphorylation Kit (T4 Polynucleotide Kinase – Biolabs), Transposomes Preparation Kit (Mu/EZ-Tn5 transposase).



**Figure 2.1** Plasmid pTnTMDH5(del)oriR6K

Map of plasmid used for genetic manipulation of *S. Typhi* and *S. Paratyphi* A

### **2.7.2.1 Plasmid digestion**

This step was included to avoid grown kanamycin resistant strains that contained an intact original plasmid, therefore guaranteeing all of the recovered organism had a Tn5 insertion. A plasmid digestion reaction was set up in a 10 $\mu$ l volume, consisting of 2.5 $\mu$ l of pTnTMDH5(del)oriR6K (10ng/mL), 1 $\mu$ l of 10X NEB4 buffer, 5.5 $\mu$ l water, 0.5 $\mu$ l MspAII restriction enzyme and 1 $\mu$ l BSA. The mixture was incubated at 37°C for 2 hours.

### **2.7.2.2 Transposon amplification**

The transposon was amplified using specific oligonucleotides (5'-CTGTCTCTTATACACATCTC CCT and 5'-CTGTCTCTTATACACATCTCTTC) with the proofreading DNA polymerase, Pfu Ultra Fusion II, (Stratagene). The reactions were established in a stock of ten and equally distributed into ten wells prior to the thermal cycling to assure homologous amplicon production. Ten reactions were established in 200 $\mu$ l mixture of 20 $\mu$ l 10X buffer, 2 $\mu$ l deoxyribonucleotide triphosphates (dNTPs, 10mM), 2 $\mu$ l digested plasmid, 0.4 $\mu$ l Primer (forward and reverse, 100nM), 171 $\mu$ l water and 4 $\mu$ l Pfu (Ultra fusion II). The thermal cycling was: 95°C for 90 seconds followed by 30 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 20 seconds and elongation at 72°C for 20 seconds, then 72°C for 3 minutes.

### **2.7.2.3 Phosphorylation**

The Tn5 amplicons were phosphorylated to obtain Pi-5' terminus products, which were able to forms transposomes in the later step. The Tn5 amplicons were purified using

Quick-Start PCR purification kit (Qiagen) following the manufacture procedure before being subjected to phosphorylation. The phosphorylation reaction was established in 80µl total consisting of 70µl purified Tn5 amplicons (approximately 50µg/ml), 8µl 10X T4 buffer, 1µl ATP (75mM, Roche) and 1µl T4 Polynucleotide Kinase (Biolabs). The mixture was incubated at 37°C for 30 – 45 minutes and inactivated at 65°C for 20 minutes.

#### **2.7.2.4 Phenol:chloroform extraction and precipitation**

The phosphorylated Tn5 amplicons were purified from proteins (T4 nucleotide kinase) and other chemicals in the phosphorylation reaction for the transposome preparation step. The extraction reaction included 2 volumes of phosphorylated Tn5 amplicons with 1 volume of phenol:chloroform. The mixture was mixed well and separated by centrifugation at 4°C at 13,000rpm for 10 minutes, and the supernatant was kept. The DNA containing supernatant was precipitated in a 30µl reaction including 10µl supernatant, 1µl NaOAc 3M pH7.5 and 19µl absolute Ethanol. The mixture was mixed well and centrifuged at 4°C at 13,000rpm for 10 minutes. The supernatant step was discarded and the pellet was rinsed twice with 200µl 70% Ethanol. The pellet (pure phosphorylated Tn5) was eluted in 10µl TE (pH7.5). The pure phosphorylated Tn5 was stored at -20°C.

#### **2.7.2.5 Transposome preparation**

Transposome preparation was always performed in 2 parallel reactions with (Tn5<sup>+</sup>) and without (Tn5<sup>-</sup>) EZ-Tn5 Transposase for the purpose of monitoring the downstream

activity of Tn5. The Tn5<sup>+</sup> reaction included 2µl pure phosphorylated Tn5 DNA (~70µg/ml in TE pH7.5), 4µl EZ-Tn5 Transposase, 2µl 100% glycerol and 0µl 50% glycerol. Accordingly, the Tn5<sup>-</sup> reaction included 2µl pure phosphorylated Tn5 (~70µg/ml in TE pH 7.5), 0µl EZ-Tn5 Transposase, 2µl 100% glycerol and 4µl 50% glycerol. The solutions were mixed well and incubated at room temperature for 30 minutes.

#### **2.7.2.6 Electrotransformation**

Sixty microliters of competent cells were mixed with 0.2mL of transposomes and stored on ice for at least 10 minutes. The cells were then electrotransformed in a 1mm electrode gap cuvette using a Bio-Rad GenePulser II setting to 1.8 kV, 25 mF, and 200 Ω. Cells were immediately recovered in 1mL of SOC medium (Invitrogen) and incubated at 37°C for 2 hours then spread on LB agar containing kanamycin (25mg/mL). After overnight incubation at 37°C, kanamycin resistant colonies were resuspended in sterilized 10% glycerol using a bacteriological spreader. This procedure was also used for electrotransformation in section 2.9.1.3. Typically, one batch of mutants (one day mutant generating) included 10 electrotransformations generating approximately 3x10<sup>5</sup> mutants.

### **2.8 Serum (plasma) bactericidal assays (SBAs)**

#### **2.8.1 Reagents**

Phosphate Buffer Saline-Calcium Chloride (PBS-CaCl<sub>2</sub>) was prepared by mixing buffer A (100mL of 0.9mM CaCl<sub>2</sub>·2H<sub>2</sub>O) into buffer B (100mL of 10X PBS, Sigma Aldrich)



and filling up to 1,000mL with double distilled water. Buffer was filtered by 0.2µm sterile membrane and stored at 4°C. SBA Diluting Buffer (prepared on ice before performing the experiment) was prepared by adding BSA (bovine serum albumin, Sigma Aldrich) into an appropriate volume of PBS-CaCl<sub>2</sub> (according to the volume of samples) to obtain a final concentration of 0.1% BSA in PBS-CaCl<sub>2</sub>. Bentonite (Sigma Aldrich) was freshly prepared daily by rinsing three times with 1mL saline (10mg/mL NaCl) for every 10mg of bentonite (centrifuge at 2,500rpm, 20 minutes, 4°C). Bentonite was used at a concentration of 1mg for every 100µl plasma.

### **2.8.2 Human complement**

SBAs were performed using human complement because baby rabbit complement (BRC) was found to be too toxic for *S. Typhi*; the natural killing rate of *S. Typhi* with BRC was >80%. For these purposes, blood was collected in silica-bead-collecting-tubes (not EDTA) as *S. Typhi* was found to be susceptible to EDTA. Blood donated from a volunteer whose plasma was demonstrated to be negative for Vi, anti-toxoid, and anti-YncE antibody) was centrifuged at 4,000rpm for 10 minutes. The raw complement containing plasma was aliquoted into smaller volumes and stored at -80°C.

Plasma containing complement still carries several elements (lysozymes) which are able to kill or inhibit the survival of bacteria. Therefore, the raw complement containing plasma was treated with bentonite in order to minimize any components that may be harmful to bacteria. Accordingly, a certain volume of raw complement containing plasma was mixed with a corresponding amount of rinsed bentonite and incubated at

37°C for 10 minutes on the day of use. The mixture was centrifuged at 2,500rpm at 20°C for 10 minutes. The bentonite-treated-complement containing plasma (supernatant) was distributed into two equal volumes. The first was kept on ice and used as active complement. The second was inactivated at 56°C for 30 minutes and used as in-active complement.

### **2.8.3 Bacterial preparation**

Bacteria (*S. Typhi* AS252/*S. Paratyphi A* 01TY546) in early log phase were used for the SBA experiment. Bacterial growth was initiated with a beginning suspension of 0.05 OD<sub>600</sub> and horizontally agitated at ~180rpm at 37°C until an OD<sub>600</sub> of 0.1. The bacterial solution was diluted in LB broth to obtain a concentration of approximately  $1.2 \times 10^6$  CFU/mL (ranged 1-2 x10<sup>6</sup> CFU/mL).

### **2.8.4 Toxicity testing**

Treated complement (or plasma samples after inactivation at 56°C for 30 minutes) was examining its toxicity for *S. Typhi* (wildtype parent strain, pool of transposon mutant library and single gene knocked out mutant strain) and *S. Paratyphi A* (wildtype parent strain, single gene knocked out mutant strain) where appropriate. The procedure for the toxicity testing was as described for full SBA assay (section 2.8.5) but including only treated complement (or treated plasma), diluting buffer and *S. Typhi*/*S. Paratyphi A*. A control assay was also performed with in-activated treated complement. An acceptable killing rate was deemed to be <30% in comparison to the control.

### **2.8.5 Serum (plasma) bactericidal assays (SBA) performance**

All tested clinical plasma samples (or immunized mice plasma samples) were heated at 56°C for 30 minutes before being subjected to SBAs to inactivate the complement components in the plasma. The dilution of plasma samples will be specified where appropriate. SBAs were performed in a 96-well plate (Thermo Scientific; individual SBA was prepared in a single well consisting of 18.5µl SBA diluting buffer, 12.5µl diluted plasma samples, 6.5µl active complement and 12.5µl diluted bacteria suspension. Parallel SBAs were performed using the same procedure but with in-active complement. Further, each SBA was performed in triplicate in three individual wells. The initial number of bacteria challenged in the assay was monitored by plating 7µl of the mixture onto LB plates, which were incubated at 37°C overnight. Colonies were enumerated (X) and calculated back to the total numbers of a whole well as  $50X/7$ . The remaining volume of the mixture was incubated at 37°C and gently agitated for 90 minutes. The numbers of bacteria were again enumerated (Y) by plating 7µl of the mixture on LB plates, which were incubated at 37°C overnight. The total number of colonies was calculated by  $Z=43Y/7$ . There were  $Z_A$  and  $Z_I$  which represent the number of bacteria after 90 minutes being challenged in a well, with Active or In-active complement, respectively. Therefore, for a specific plasma sample, the killing rate was calculated as  $1-(Z_A/Z_I)$ . The killing rate could be calculated on the average number or the total number of the triplicate.

## **2.8.6 Bactericidal assays specific for Chapter 6**

### **2.8.6.1 Bactericidal assays on *Salmonella* Typhi transposon mutant library**

The *S. Typhi* transposon mutant library consisted of approximately  $5 \times 10^5$  individual colonies. Therefore, the number of unique insertion sites across the whole *S. Typhi* genome was predicted to be  $2 \times 10^5$ , (2/5 the obtained mutant colonies), which was approximately twice as high as the rate of unique insertion sites over the number of the mutant library obtained from previous transposon mutant library of the *S. Typhi* Ty2-derived CVD908-htrA ( $2 \times 10^5$  unique insertion sites over  $10 \times 10^5$  mutant colonies)<sup>343</sup>. To challenge every mutant (carrying unique insertion site) at least 10 times,  $2 \times 10^6$  bacteria of the library required to be screened. The final bacterial concentration in SBA was approximately  $1.5 \times 10^4$  CFU/reaction of 50  $\mu$ l ( $3 \times 10^5$  CFU/mL), which was 1:4 diluted from the bacterial concentration prior SBA (see section 2.8.3 and 2.8.5). Consequently, to challenge every mutant at least 10 times, 133 reactions needed to be performed ( $133 \times 1.5 \times 10^4 \sim 2 \times 10^6$ ). However, as the SBAs were performed in 96-well plates, I performed the challenge assay on two complete 96-well plates, which resulted in every mutant (carrying unique insertion site) being challenged approximately 14 times.

Every plasma pool (see section 2.8.6.1.2) collected from each of the four time points post admission was subjected to the SBA using the *S. Typhi* transposon mutant library on two full 96-well plates (2 plates x 4 time points  $\sim$  8 plates). The experiment was performed in parallel with control experiments where the same pool of plasma was

assayed on wildtype parent strain of the transposon mutant library (10 wells for every pool of plasma).

#### **2.8.6.1.1 Harvesting colonies recovered from bactericidal assays**

The recovered colonies from the bactericidal assays using the transposon mutant library (individually for each time point) were scraped using sterile glass slides and suspended in PBS in a 50mL tube. The suspended bacteria were subjected to extraction of total genomic DNA (Wizard Kit, Promega). Purified nucleic acid was stored at -20°C until being sent to be Wellcome Sanger Institute (Hinxton, Cambridge, UK) for sequencing using their Transposon Directed-Insertion Site Sequencing (TraDIS) protocol. The original transposon mutant library was TraDIS simultaneously.

#### **2.8.6.1.2 Titrating the plasma samples for bactericidal assays**

A plasma pool (at every time point) used to challenge *S. Typhi* transposon mutant library was required to be diluted to the optimum concentration to kill/inhibit the growth of *S. Typhi* wildtype parent strain during the bactericidal assay. Accordingly, plasma samples from six patients that had been pre-tested for their bactericidal activity to *S. Typhi* wildtype parent strain were diluted with PBS to obtain dilutions as listed in Table 2.5.

**Table 2.5** Working dilutions of plasma prior to SBA challenging the *S. Typhi* transposon mutant library

Patient ID	Dilution			
	Day 1	Day 8	Month 1	Month 3
01TY305	1:50	1:25	1:25	1:25
01TY311	1:100	1:25	1:25	1:50
01TY447	1:50	1:75	1:50	1:25
01TY473	1:25	1:37.5	1:12.5	1:37.5
01TY405	1:25	1:50	1:25	1:25
01TY410	1:37.5	1:37.5	1:37.5	1:25

#### **2.8.6.2 Bactericidal assays on *Salmonella* Typhi and *Salmonella* Paratyphi A mutants**

Thirteen *S. Typhi* antigens including six antigens were recognised by both the protein array and TraDIS (STY1372, STY1498\_*hylE*, STY1612, STY1767\_*nlpC*, STY3208, STY4190\_*yhjJ*,) and other seven antigens (STY0452, STY0796, STY1086, STY1522, STY1703, STY1886, STY4539) were purified following the procedure as described in section 2.4. These thirteen proteins (and STY1479\_*yncE*, the Vi polysaccharide and PBS) were used to immunised female C57BL/6 mice (3 for each antigen). Mice were immunised subcutaneously at a dose of 100µg per mouse per antigen and adjuvanted with labile toxin. Mice were boosted on day 7 and day 21 with the same dose and killed by cardiac puncture under terminal anaesthesia on day 28. Blood was drained using a ventricular bleed immediately and separated into cells and plasma by centrifugation at 16g for 5 minutes.

Individual pairs of *S. Typhi* AS252/*S. Paratyphi* A 01TY546 wildtype (WT) and the specific isogenic descendant mutant (dMT) were challenged by bactericidal assay (following the same procedure as described in section 2.8.5) using plasma extracted from mice that had been immunized with the specific antigen. All fifteen plasma samples from the immunised mice were used to challenge *S. Typhi*, while only four plasma samples (STY0796, STY1086, STY1372, and STY1498) were used to challenge *S. Paratyphi* A. The dilution of immunised mice plasma was 1:12.5. Control bactericidal assays were performed with plasma from unimmunized mice. The rate of bacterial killing calculated as described in section 2.8.5; the presented killing rates were

magnitudes of the killing rate of antigen-immunised mice plasma (WT strain and an isogenic dMT strain individually) subtracted by killing rate of the control mice plasma (WT strain and an isogenic dMT strain individually).

The immunization of mice was performed by Dr Simon Clare at Wellcome Sanger Institute, Cambridge, UK. Protein STY1479\_ *yncE* was provided by Professor Edward T. Ryan, Harvard University, USA. Vi polysaccharide was provided by Novartis Vaccines for Global Health.

#### **2.8.7 Bactericidal assay for Chapter 7**

Plasma bactericidal assays (SBA) in Chapter 7 were performed using *S. Typhi* AS252. I used a collection of plasma samples collected from 4 patients with culture confirmed acute typhoid fever on day 8 after admission, from 3 culture confirmed *S. Typhi* carriers, and from 4 cholecystectomy controls. Additionally, plasma collected from 5 samples from the plasma bank that were indicative of typhoid carriage and 7 samples from the plasma bank that had a profile not associated with carriage were also assayed. Bactericidal activity to *S. Typhi* of any plasma sample was examining via 2-fold serial dilutions starting from 1:100 (to 1:12,800) of plasma.



## **2.9 Direct homologous recombination for Chapter 6**

### **2.9.1 Primer design**

Primers for bacterial mutagenesis were designed using Artemis (The Wellcome Trust Sanger Institute, UK), for site-specific recombination. The designed primers targeted the candidate loci and were 40-50bp in length with a GC content of 40-50%. Eighteen pairs of primers were constructed for site-specific mutagenesis. Forward primers were tagged with 'TGTGTAGGCTGGAGCTGCTTCG' and reverse primers were tagged with 'CATATGAATATCCTCCTTA'. These 19- to 22- nucleotide extensions are homologous to regions adjacent to the antimicrobial resistance gene in the pKD4 plasmid for site-specific mutagenesis. Further, primers were also designed to check successful homologous recombination. The designed primer sets were synthesised by Sigma and are shown in Table 2.6.

**Table 2.6** Primer design for gene knock-outs in *Salmonella* Typhi and *Salmonella* Paratyphi A

Primer ID	Sequence	String	Position	Length	GC%
STY0452-F	acgctccgccagaacgtttattgattaacaggctgaatatcatggatcggTGTGTAGGCTGGAGCTGCTTCG		460891..460940	50	46
STY0452-R	actaccattacgctatcggaccacgaaggaaatgatcgacatgacaagacgCATATGAATATCCTCCTTA	complement	461437..461487	51	47
STY0452-R-check	gaaacaaaatgggcaatgcatacggcgctctgaccagatgatttagg	complement	461573..461619	46	47
STY0796-F	tgagcagtaacttcagacatcacctgttgagctctgctgttactggttggcTGTGTAGGCTGGAGCTGCTTCG		792520..792569	50	48
STY0796-R	aatatcccgctggcaacggcgccatataattactgatttcagatttgagcgcCATATGAATATCCTCCTTA	complement	793396..793445	50	46
STY0796-R-check	tgagtttgatgttaaattagtgggctgctgcaggattcgaacctgcgacc	complement	793516..793564	49	47
SPA1993_STY0796-F	tgagcagtaacttcagacatcacctgttgagctctgctgttactggttggcTGTGTAGGCTGGAGCTGCTTCG	complement	2067828..2067877	50	48
SPA1993_STY0796-R	aatatcccgctggcaacggcgccatataattactgatttcagatttgagcgcCATATGAATATCCTCCTTA		2066952..2067001	50	46
SPA1993_STY0796-Fcheck	gcggcttacgctaagaacgcgcgctgtactggttactaagagaattg	complement	2067880..2067929	50	50
STY1086-F	ataaaagctattatcagctcccgatagcgcaaaagcgggtgtgcaaaagcaccTGTGTAGGCTGGAGCTGCTTCG		1065926..1065975	50	48
STY1086-R	tgatgcgccgtcaatgcgcaaaattcacaccaatgtcataatttgagcCATATGAATATCCTCCTTA	complement	1066544..1066593	50	46
STY1086-R-check	ctcgtttttgtctcctcatgctcgtttccctcatattttgtatctggtgg	complement	1066648..1066697	50	46
SPA1019_STY1086-F	ataaaagctattatcagctcccgatagcgcaaaagcgggtgtgcaaaagcaccTGTGTAGGCTGGAGCTGCTTCG	complement	1836571..1836620	50	48
SPA1019_STY1086-R	ctttatctcagggaaggcgctttatctcatctcgatagcggctgttcCATATGAATATCCTCCTTA		1836113..1836162	50	50
SPA1019_STY1086-Fcheck	gaaaaaatggctagtcgtgataatggcggttctggctgcgtcatgtagctcc	complement	1836631..1836682	52	50
STY1372-F	ggccatcccgtaaccattttgtgtgtgtgttaccgatttggctgtggTGTGTAGGCTGGAGCTGCTTCG		1328119..1328170	52	46
STY1372-R	tctctccagttcggatgctctgcacgaagaatattccacgcctgaatgcCATATGAATATCCTCCTTA	complement	1328268..1328319	52	50
STY1372-R-check	ggtaaaaaacgcccagcccaagaaatcgacagcagcaccagaatacg	complement	1328437..1328484	48	47
SPA1195_STY1372-F	ggccatcccgtaaccattttgtgtgtgtgttaccgatttggctgtggTGTGTAGGCTGGAGCTGCTTCG		1328119..1328170	52	46
SPA1195_STY1372-R	tctctccagttcggatgctctgcacgaagaatattccacgcctgaatgcCATATGAATATCCTCCTTA	complement	1328268..1328319	52	50
SPA1195_STY1372-R-check	ggtaaaaaacgcccagcccaagaaatcgacagcagcaccagaatacg	complement	1328437..1328484	48	47
STY1498-F	atgtatcagacgtcaggaacctcgaaaacgctcttcttaccgtgtcttgtggTGTGTAGGCTGGAGCTGCTTCG		1455050..1455103	54	46.3
STY1498-R	aatatctcagaacaaactgtagaggtagttaaaagcgcgatcgaaaccgcagatggCATATGAATATCCTCCTTA	complement	1455902..1455958	57	42.1
STY1498-Rcheck	ctcaaaactgttgatcagggtatcgaaattgtgccgggtaccg	complement	1456312..1456353	42	47.7
SPA1306_STY1498-F	aaaagagttaagccgttttaaacaggagtactcgcaggaagcttctTGTGTAGGCTGGAGCTGCTTCG	complement	1381576..1381620	45	42.22
SPA1306_STY1498-R	tattcattacaggtgttaatacttttcttgcagctccttttagtaCATATGAATATCCTCCTTA		1381338..1381383	46	30.43
SPA1306_STY1498-Rcheck	actttcgatcaaatcatccagaaaagcaaaagccgggtgccgggtat		1381073..1381118	46	47.83
STY1522-F	gaccaggttttatagttgatatcattgtagcgcgcaccatcatctcaccTGTGTAGGCTGGAGCTGCTTCG		1478652..1478701	50	46
STY1522-R	tttcattggcgctacgtcggaatatatcaggatttacccatcatggttaacCATATGAATATCCTCCTTA	complement	1479522..1479571	50	44
STY1522-R-check	tggtcacacggataacgcgtttcatgttagttcgtgtgtttaactctcc	complement	1479806..1479855	50	46
STY1612-Fe	ttgaattctgacggtagtgagttttctgctaccttcattgggtgttgTGTGTAGGCTGGAGCTGCTTCG		1549221..1549271	51	47
STY1612-Re	acttcagtttttataaagcgcgtccagtttggcctcaatgactgtctggCATATGAATATCCTCCTTA	complement	1549450..1549500	51	47
STY1612-R-check	tctccagatcaaccattttacggtcgaccagataaccagttcg	complement	1549686..1549729	44	45
STY1703-F	atctccagggaattatatacccatatgcagcaactgagactccagcTGTGTAGGCTGGAGCTGCTTCG		1629028..1629075	48	46
STY1703-R	aacgtatggactttgaacaactcatgcaccaggcattaccattggtgCATATGAATATCCTCCTTA	complement	1629254..1629301	48	46
STY1703-R-check	tggataagaaacaacaaatggcggactattcactcaggcgcagagc	complement	1629502..1629548	47	48
STY1767-F	ccagtcactacggacggaaaacagacgtaacttcagatactaattgaagcgTGTGTAGGCTGGAGCTGCTTCG		1689925..1689974	50	46

STY1767-R	catgcgttttggcttctgttatcacagcattgttctctggcaggatgcCATATGAATATCCTCCTTA	complement	1690620..1690668	49	47
STY1767-R-check	tftatgtcaggcaggaaftgcgattgtgatgtagccacgatctgaacc	complement	1690911..1690960	50	46
STY1886-F	ctgcgctaataatcagtgactacaagttatgacctggaatcttcagggtcttctcagcTGTGTAGGCTGGAGCTGCTTCG		1785447..1785503	57	45
STY1886-R	ttcgtgcaaaaaggctacgggataatgatcagaagcgagttgtggattacgCATATGAATATCCTCCTTA	complement	1786138..1786189	52	46
STY1886-R-check	tgatacaggtagtcatgaatgattgccgctttggcatattcaccatccgg	complement	1786444..1786493	50	46
STY3208-F	atttcagtgaaatcgacggcaatattaaatgacggaccaacaacccattcggTGTGTAGGCTGGAGCTGCTTCG		3073414..3073464	52	43
STY3208-R	tgcaatggcagctaaccggagaaaaataatccagtaatctattagtgaccCATATGAATATCCTCCTTA	complement	3074103..3074153	51	43
STY3208-R-check	tgttctgctcctgcgtaccgattagcgactaccagtaatttactgattcg	complement	3074647..3074696	50	46
STY4190-F	attaccaggattgtgatcagggttcacatgcagggcacaaaaattcgTGTGTAGGCTGGAGCTGCTTCG		4053338..4053386	49	44
STY4190-R	gaaccatgatttcatcccacgtcgcctttaacgcctcatataaattccggCATATGAATATCCTCCTTA	complement	4054732..4054783	52	44
STY4190-R-check	ttccggcagtaaaactgccgaaagagaaagtgatagacaccactg	complement	4055057..4055101	45	47
STY4539-F	tcaaccagctcatcgatattactatgccagggcgaactggttaactcagtcTGTGTAGGCTGGAGCTGCTTCG		4408732..4408781	50	46
STY4539-R	ccgtctcaaagttgcctttaataccaacgggtgcgtcaatgcggaatccCATATGAATATCCTCCTTA	reverse complement	4409600..4409649	50	48
STY4539-Fcheck	ttacgggtcatccacgggattttcatgaacacacaagcgcttttctgcct		4408494..4408543	50	46
STY4294-F (ompR-F)	ttataagattctgggtgtgatgacgatatgcgtctcgccgctactggTGTGTAGGCTGGAGCTGCTTCG		4170277..4170326	50	50
STY4294-R (ompR-R)	gtcaacctcttcaccttcgccgtgaccataatgatcgccattggattacCATATGAATATCCTCCTTA	complement	4170486..4170535	50	51
STY4294-Fcheck (ompR-Fcheck)	ttgcgcacacgggggtataacgtgatcgtcccagacagaataaataatgggg		4170068..4170117	50	50

STY and SPA are prefix of a CDS on the *S. Typhi* genome CT18 and *S. Paratyphi A* genome ATCC9150, respectively

F: Forward primer

R: Reverse primer

F-check: Checking primer on the same genome string as forward primer, using the same reverse primer of the selected locus

R-check: Checking primer on the same genome string as reverse primer, using the same forward primer of the selected locus

### 2.9.2 Amplification of the pKD4 Kan<sup>R</sup> gene cassette

Amplification of a 1,749 bp length Kan<sup>R</sup> gene cassette in pKD4 was performed in ten reactions. A stock of 200µl PCR reaction mixture containing 20µl 10X buffer, 4µl dNTPs (10mM), 0.8µl Primer (forward and reverse primers shown in Table 2.6, 10nM), 5µl of *Pfu* polymerase (Aligent, US) and 1µl of purified DNA plasmid pKD4 was generated. The thermal cycling conditions were: denaturation at 95°C for 90 seconds followed by 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 20 seconds and elongation at 72°C for 2 minutes. A 10-minute elongation was added after the final cycle. PCR amplicons were visualised using Ultraviolet (UV) transilluminator (Bio-Rad) with the Quantity One (Bio-Rad) imaging software on 1% agarose gel (Bio-Rad) after staining with 1% Nancy (Sigma). PCR amplicons were purified using Quick-Start PCR purification kit (Qiagen) for electrotransformation.

### 2.9.3 Homologous recombination (site-specific mutagenesis)

Homologous recombination was performed using *S. Typhi* strain AS252 (or *S. Paratyphi* A 01TY546), which was firstly transformed with plasmid *psim18*. Competent cell preparation and electrotransformation were performed as described in sections 2.7.1 and 2.7.2.6, respectively. However, the selection for *psim18* plasmid was hygromycin and the *psim18*-electrotransformed *S. Typhi* cells were recovered and incubated at 30°C until being harvested (*psim18* plasmid is maintained at 30°C). *Psim18* plasmid carries an operon encoding recombinase (induced at 42°C) which facilitates recombination. Therefore, *S. Typhi* (or *S. Paratyphi* A) carrying *psim18* could accept purified PCR amplicons (section 2.9.1.2) for recombineering (the pKD4 Kan<sup>R</sup> gene cassette).

Competent cell preparations of *S. Typhi* (or *S. Paratyphi A*) carrying *psim18* were handled as described in section 2.7.1 with the additional step of recombinase induction. Accordingly, when the bacterial growth was in early exponential phase (OD at 600nm ~0.3 - 0.4) the recombinase was induced by incubation at 42°C in a water bath for 15 minutes before being transferred onto ice and ready for subsequent washes. *S. Typhi* (or *S. Paratyphi A*) carrying *psim18* was electrotransformed with a piece of nucleotide sequence generated from section 2.9.1.2 using the same procedure as described in section 2.7.2.6.

#### **2.9.4 Examination the successful recombination**

Site-specific mutants were examined by colony PCR. The amplification conditions were as described in section 2.7.2.2, except template material was bacteria scraped directly from the mutant colonies. To verify the newly recombineering sequences at the target locus, the sequences flanking the target locus (at the 5' end or 3' end) were sequenced. The flanking sequence of the *pkD4 Kan<sup>R</sup>* cassette was amplified using specific primers (Table 2.6) and the amplification condition as described in section 2.7.2.2. PCR amplicons were purified using Quick-Start PCR purification kit (Qiagen) and Sanger sequenced. Each 10µl of the amplification reaction contained 1µl of purified amplicon, 2µl of 10X buffer (Applied Biosystems, California, US), 1µl of Bigdye (Applied Biosystems), 0.3µl of either forward primer with paired reverse checking primer (or forward checking primer paired with reverse primer) (Table 2.6) and 5.7 µl of water. PCR reactions were cycled at 95°C for 2 minutes followed by 30 cycles of 95°C for 10

seconds, 55°C for 5 seconds and 60°C for 4 minutes. Ten minutes at 72°C was added at the end. The whole volume of amplified products was precipitated on ice with 1µl of 3M Sodium Acetate (NaOAc) (Sigma), 1µl of Ethylenediaminetetraacetic acid (EDTA) (Prolabo, Pennsylvania, US) and 35µl of 100% ethanol (Merck). The mixture was vortexed and centrifuged at 4°C at 14,000rpm for 15 minutes, and supernatant was discarded. The pellet was washed twice with 500µl of 70% ethanol by centrifugation and the supernatant was discarded. Samples were air-dried, removed from ice and re-suspended with 10µl of Hidi (Applied Biosystems). The sequences were analysed using an Applied Biosystems genetic analyser, sequences were aligned in Artemis against the *S. Typhi* CT18 (or *S. Paratyphi* A ATCC9150) genome and the pKD4 plasmid sequence to confirm homologous recombination.

## **2.10 Cytokine measurement**

A collection of plasma samples including 10 from *S. Typhi* carriers, 17 from age-matched cholecystectomy controls, 89 from aged-matched *S. Typhi* acute enteric fever (Day 8 after admission) were submitted to measure eight pro-inflammatory cytokines (IL-1, IL-2, IL-5, IL-6, IL-12, IL-13, TNF-alpha and Interferon (IFN)-gamma) and two anti-inflammatory cytokines (IL-4 and IL-10) using Precision Pro (Bio-plex). The performance followed the recommendations of the manufacture. Briefly, a pre-wet filter plate was loaded with diluted beads (50µl per well) and washed twice with wash buffer (100µl per well) using a washing station. Prepared (diluted) standards and samples were added into separate wells (50µl per well). The plate was wrapped, agitated at 1,100rpm for 30 seconds then maintained at 300rpm for 30 minutes in the dark, then washed 3

times with wash buffer. Prepared detection antibody was added (25µl per well). The plate was sealed and agitated at 300rpm for 30 minutes in the dark. Ready Streptavidin-PE (50µl per well) was added and the plate was sealed, incubated and washed as previous steps. Cytokine bound beads were diluted in assay buffer (150µl per well). The plate was sealed again and rigorously mixed at 1,100 rpm for 30 seconds, unsealed and read immediately on the Bio-plex system.

## **2.11 Data analysis**

All analysis were performed using R software (version 3.3.1; R Foundation for Statistical Computing) <sup>344</sup>. All confidence intervals (CIs) are reported 2-sided at the 95% intervals; all significance testing was performed 2-sided with a significance level of  $\rho$  value  $\leq 0.05$ .

### **2.11.1 Data analysis for Chapter 4**

A geometric mean optical density was calculated to summarize the IgM response to the *S. Typhi* antigens in each arm of validation group including the negative reference population samples (afebrile controls and other confirmed febrile infections) and the positive reference population (febrile patients with confirmed *S. Typhi*). The Wilcoxon signed-rank test was used to test the null hypothesis; no difference in optical densities between the patient groups. Spearman's rho was used to investigate potential correlations between IgM antibody responses against the various antigens. Receiver operating characteristic (ROC) curves were used to determine the optimal cut-off and the specificity and sensitivity of the various antigens. A performance estimation of more than one antigen combination was evaluated using Support Vector Machine (SVM). SVM is a supervised learning model that analyses data for classification and regression analysis using a training and testing data set. All analyses were performed with R software (version 3.3.1; R Foundation for Statistical Computing). All confidence intervals (CIs) are reported 2-sided at the 95% intervals; all other significant tests were performed 2-sided with a significance level of  $\rho$  value  $< 0.05$ .



The SVM was performed with assistance from Mr. Tran Tuan Anh (Enteric Infections Group, OUCRU, Vietnam) in order to identify combinations of antigens that could diagnose acute typhoid fever.

### **2.11.2 Data analysis for Chapter 5**

A geometric mean of EU was calculated to summarize the antibody responses (IgM and IgG) where appropriate against typhoid toxin, Vi and O2 antigen in patient group, the community controls, and the plasma bank. The Wilcoxon signed-rank test and Wilcoxon matched-pairs signed-rank test were used to test the null hypothesis that there was no difference in EU between groups and between time points, respectively. The classification of enteric fever cases from unidentified febrile patients was evaluated using SVM.

For assessing diagnostic classification, the acute antibody profile of anti-toxin IgM alone or combined with anti-Vi IgM and anti-O2 IgM from patients with a confirmed *S. Typhi* or *S. Paratyphi A* infection was used as positive control; the same profile from febrile patients with an unknown pathogen was used as the negative control. Significant diagnostic associations were calculated by Castro and Azevedo <sup>345</sup> approach where the entire longitudinal antibody profile, individually, was discretized into breaks <sup>346</sup>. The significant dynamic patterns were then statistically calculated using a hidden Markov model and binomial distribution (insignificant patterns were not used for later calculation). Sero-conversion was considered when a significant pattern showed antibody titre of any later time points elevated at least one break in comparison to the

baseline antibody titre. A mixed effect model was utilized for longitudinal typhoid toxin IgG profile <sup>347,348</sup>. K-mean clustering was employed for the association between anti-toxin IgG versus anti-Vi IgG and anti-toxin IgG versus anti-O<sub>2</sub> IgG <sup>349,350</sup>.

The mixed effect model and K-mean clustering were performed with assistance from Mr. Tran Tuan Anh.

### **2.11.3 Data analysis for Chapter 6**

#### **2.11.3.1 TraDIS library analysis**

Approximately 2 million reads per sample were generated. Illumina FASTQ files containing 50 base pairs reads were parsed for 100% identity to the transposon. The tags were stripped from the resulting reads and mapped back onto *S. Typhi* CT18 (Acc. No. AL513382\_ using SMALT-0.7.2) and the precise insertion site of the transposon was determined as described previously <sup>343,351,352</sup>. Briefly, for each gene (CDS) the insertion index, the number of insertions in each gene divided by the total gene length, was calculated by excluding 10% of the 3' end of gene, which could be functional despite having an insertion. To determine the genes required for serum sensitivity, the input transposon library was compared to the serum challenged library (output) and significant differences in insertion counts were expressed as Log<sub>2</sub>Fold-Change <sup>352</sup> and  $\rho$  values were corrected for multiple testing using the Benjamini-Hochberg method. Only genes (CDSs) with a  $\rho$  value (Q value) of <0.1 (a hypothetical 10% false discovery rate [FDR]) and an absolute log fold change (logFC) of >2 were considered significant. However, for a higher specificity, I only selected genes (CDSs) with a  $\rho$  value <10<sup>-5</sup>.

The candidate gene collections were classified according to functional classes based on the Sanger Institute classification system (<http://www.sanger.ac.uk>)<sup>98</sup> (Table 2.7). Additionally the gene collections were also categorised into various biological pathways using the KEGG pathways<sup>417</sup>.

The primary analysis on the sequencing data to define the collection of genes that have  $\log_{2}FC > 2$  and with a  $p$  value  $< 0.05$  was performed by Dr Christine Boinett (Enteric Infections Group, OUCRU, Vietnam).

**Table 2.7** *Salmonella* Typhi gene functional classification scheme

1.A Degradation	1.D.4 Aromatic Amino Acid	1.G.9 Riboflavin	4.A.2 Transport Cations
1.A.1 Degradation of carbohydrate	1.D.5 Histidine	1.H Fatty acid biosynthesis	4.A.3 Transport Carbohydrates, organic acids, alcohols
1.A.2 Degradation of amino acids	1.D.6 Pyruvate Family	2 Broad regulatory function	4.A.5 Transport Anions
1.B Energy metabolism	1.E Polyamine synthesis	3.A Macromolecule synthesis/modification	4.A.6 Transport Other
1.B.1 Glycolysis	1.F Nucleotide biosynthesis	3.A.10 Polysaccharides - (cytoplasmic)	4.B Chaperones
1.B.10 Glyoxylate Bypass	1.F.1 Purine ribonucleotide biosynthesis	3.A.11 Phospholipids	4.C Cell division
1.B.2 Pyruvate dehydrogenase	1.F.2 Pyrimidine ribonucleic	3.A.2 Ribosomal proteins - synthesis, modification	4.D Chemotaxis and mobility
1.B.3 Tricarboxylic acid cycle	1.F.3 2'-Deoxyribonucleotide metabolism	3.A.3 Ribosomes - maturation and modification	4.G Detoxification
1.B.5.b Non-oxydative branch	1.F.4 Salvage of nucleosides and nucleotides	3.A.5 Amino acyl tRNA synthesis; tRNA modification	4.H Cell Killing
1.B.6 Entner-Doudoroff pathway	1.F.5 Miscellaneous	3.A.7 DNA - replication, repair, restriction/modification	4.I Pathogenicity
1.B.7.a Aerobic Respiration	1.G Biosynthesis of cofactors, carriers	3.A.8 Protein translation and modification	5.A IS element, Phage related
1.B.7.b Anaerobic Respiration	1.G.1 Biotin	3.A.9 RNA synthesis, modification	5.B Colicin-related function
1.B.7.c Electron Transport	1.G.10 Thioredoxin	3.B Macromolecule degradation	5.D Drug/Analogue sensitivity
1.B.8 Fermentation	1.G.11 Menaquinone	3.B.1 Degradation of RNA	5.F Adaptions and atypical conditions
1.B.9 ATP-proton motive force	1.G.12 Heme and porphyrin	3.B.2 Degradation of DNA	5.H Hypothetical protein
1.C Central intermediary metabolism	1.G.13 Cobalamin	3.B.3 Degradation of proteins, peptides, glycoproteins	5.I Unknown
1.C.2 Gluconeogenesis	1.G.14 Iron uptake and storage	3.B.4 Degradations of polysaccharides	
1.C.3 Sugar-nucleotide biosynthesis, conversions	1.G.2 Folic Acid	3.C Cell envelope	
1.C.4 Amino sugars	1.G.3 Lipoate	3.C.1 Membranes lipoprotein	
1.C.5 Sulphur Metabolism	1.G.4 Molybdopterin	3.C.2 Surface polysaccharides & antigens	
1.D Amino Acid Biosynthesis	1.G.5 Pantothenate	3.C.3 Surface structure	
1.D.1 Glutamate Family	1.G.6 Pyridoxine	3.C.4 Murein sacculus, peptidoglycan	
1.D.2 Aspartate Family	1.G.7 Pyridine nucleotide	4.A Transport/binding proteins	
1.D.3 Serine Family	1.G.8 Thiamine	4.A.1 Transport amino acid and amines	

### **2.11.3.2 Testing the difference in killing rates between the wild type parent strain and mutant descendant strain**

The null hypothesis was that there was no difference in killing rates between wild type parent strain and mutant descendant strain after being challenged with plasma from the antigen immunised animals. The null hypothesis was tested using Wilcoxon signed-rank test.

### **2.11.3.3 Testing the difference in titres of antibody IgG against a certain antigen among assessing groups and among time points**

A geometric mean of EU was calculated to summarize the antibody IgG responses against *S. Typhi* antigens in patient groups and afebrile controls. The null hypothesis that there was no difference in EU between groups and between time points was tested using Wilcoxon signed-rank test and Wilcoxon matched-pairs signed-rank test, respectively. Correlation between each pair of anti-antigen antibody was calculated using Spearman's rho Test.

## **2.11.4 Data analysis for Chapter 7**

### **2.11.4.1 Testing for significant difference in cytokine and IgG concentration and the bactericidal capacity of plasma samples**

The null hypothesis that there was no difference in cytokine level (pg/ml), antibody IgG measurement (OD), or bactericidal capability (killing rate %) between groups was tested using Wilcoxon signed-rank test.

#### **2.11.4.2 Principal component analysis (PCA) for cytokine measurement among patient groups**

Data generated from the measurements of pro-inflammatory (and anti-inflammatory) cytokines was visualised by a PCA plot. The whole set of data was produced from 10 cytokine values of each individual from every group of patients. Therefore, the whole set of data was originally on 10 dimensions, which is impossible to manually analyse. PCA analysis reduces the number of data dimensions to 2 in order to simplify the way to interpret the data. PCA was performed using R software (version 3.3.1; R Foundation for Statistical Computing).

#### **2.11.4.3 Estimating the prevalence of *Salmonella* Typhi carriage in Kathmandu**

The IgG antibody against three antigens (YncE, toxoid, and Vi) generated from *S. Typhi* carriers and cholecystectomy controls were individually tested for a best fit model using Akaike Information Criterion estimator. The log-normal model was found to best fit the data. For each antigen, density for distribution was estimated to construct the log-normal model. The estimated values of density corresponding to IgG antibody titre from the models were used to calculate global/combined odds of being *S. Typhi* carriers (pseudo likelihood of being *S. Typhi* carriers). The global odds were subsequently used to construct a ROC curve to assess the sensitivity and specificity for classification of *S. Typhi* carriers from cholecystectomy controls. The global odd thresholds of being a *S. Typhi* carrier, corresponding with different compromises of sensitivity and specificity, were used to estimate the prevalence of *S. Typhi* carriers in the anonymous individuals from the plasma bank of  $20 \leq \text{aged} \leq 65$  years. The obtained prevalence of *S. Typhi*

carrier was then extrapolated to estimate the total number of *S. Typhi* carriers in Kathmandu within the age group of  $20 \leq \text{aged} \leq 65$  years, taking proximal population figures from the 2011 government census (Table 2.8).

The statistical estimation of *S. Typhi* carriage prevalence in Kathmandu was performed with assistance from Dr Thibaut Jombart (London School of Hygiene and Tropical Medicine).

**Table 2.8** Kathmandu valley year 2011 government census

Age group	Number of people	Percent
0-4	111,600	6.4
5-9	137,162	7.9
10-14	165,679	9.5
15-19	202,174	11.6
20-24	233,794	13.4
25-29	202,417	11.6
30-34	162,746	9.3
35-39	134,021	7.7
40-44	106,558	6.1
45-49	78,678	4.5
50-54	60,754	3.5
55-59	43,354	2.5
60-64	34,946	2
65-69	25,950	1.5
70-74	18,205	1
75-79	13,220	0.8
80-84	7,482	0.4
85-89	3,660	0.2
90-94	1,314	0.1
95+	526	0



## **Chapter 3 The Control of Typhoid in Vietnam**

### **3.1 Introduction**

Typhoid fever, the disease caused by *Salmonella enterica* serovar Typhi (*S. Typhi*), is a diminishing public health problem in Vietnam<sup>81</sup>. However, the disease remains an ongoing public health issue in other parts of South and Southeast Asia<sup>353–355</sup>, and an enhanced understanding of disease estimates and the influence of antimicrobial resistance (AMR) on disease presentation are needed to better control this disease across the region. Furthermore, insights into the trends of typhoid and factors that directly impinge on disease incidence are important for allocating resources for reducing the burden of disease<sup>356</sup>. Currently, Vietnam represents an exemplar Asian country that has all but eliminated this once common infection, and there is probably much to be learnt from the control of typhoid in Vietnam. However how reduction in typhoid was precisely achieved is unclear and providing a roadmap for typhoid reduction in similar settings is largely dependent on good historical quantitative data.

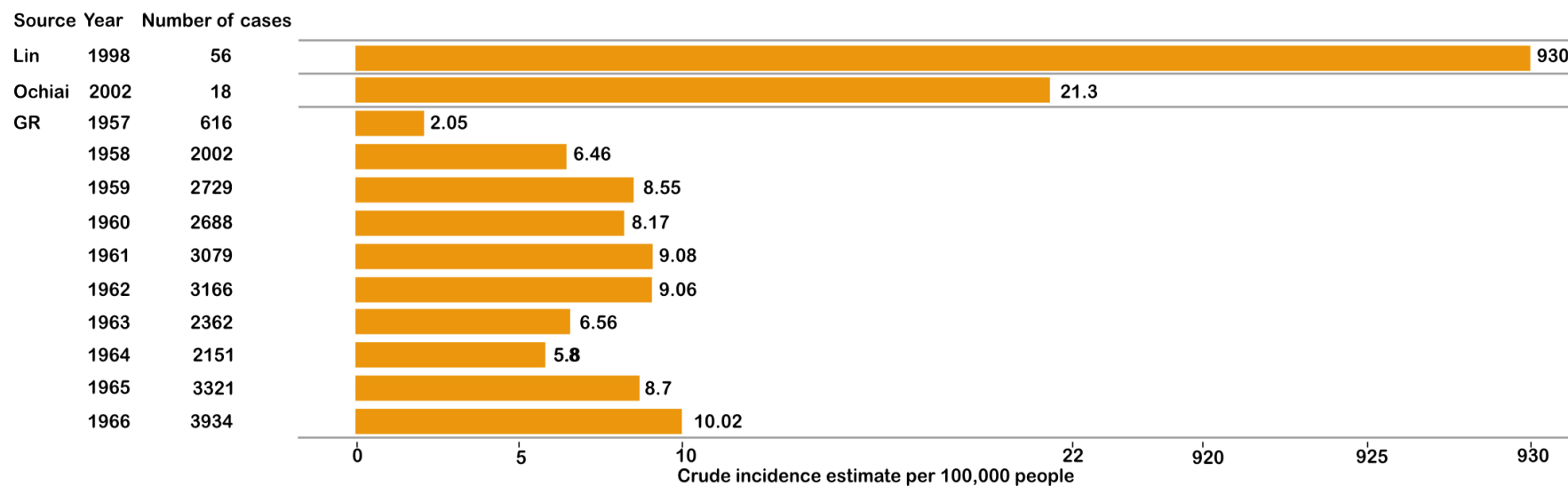
### **3.2 Typhoid fever incidence in Vietnam**

Typhoid fever has likely been endemic in Vietnam for some time, although historical incidence data for this common cause of febrile disease from across Southeast Asia before the reunification of Vietnam in 1975 is scarce. Notified typhoid fever cases reported to the pre-reunification government of South Vietnam showed a generally increasing trend from 2.05 cases per 100,000 people annually in 1957 to 10.02 cases per 100,000 people annually in 1966 (Figure 3.1)<sup>357</sup>. Health care provisions, and water and sanitation infrastructure in South Vietnam during this

time period were generally poor, which likely contributed to the increasing rates of typhoid fever and other infectious diseases during this period, which were frequently observed in military personnel returning back to the United States <sup>358</sup>. By contrast, a report suggests that the pre-reunification government in North Vietnam prioritized health care access and began mass vaccination campaigns against typhoid fever and other communicable diseases as early as 1954, although reliable incidence data from North Vietnam during this period are not available <sup>359</sup>.

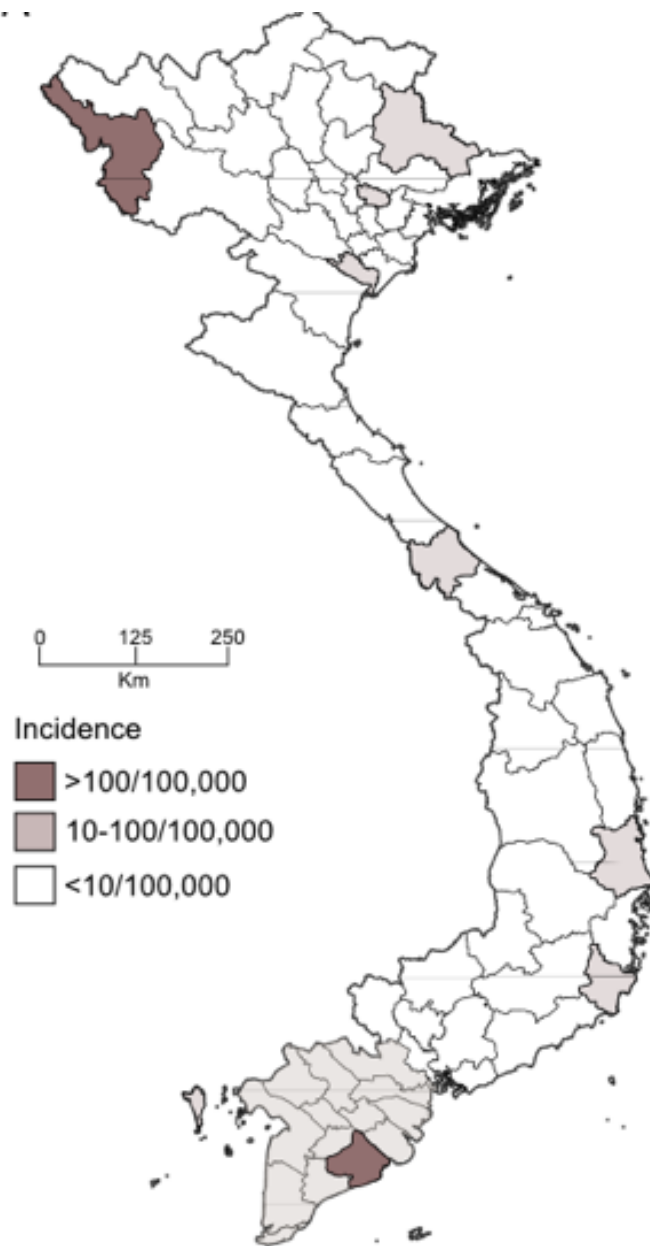
The best and most accurate recent estimates of typhoid incidence in Vietnam were calculated during the International Vaccine Institute's (IVI) Diseases of the Most Impoverished (DOMI) program, which was conducted between 1999 and 2003 <sup>360</sup>. The annual incidence of typhoid fever in Hue, in central Vietnam, in 2002-2004 was estimated to be 21.3/100,000 person years and 24.2/100,000 person years in children aged 5-15 in years (Figure 3.1). This programme went on to conduct various epidemiological investigations and vaccine studies the same location <sup>361,362</sup>. In addition, the National Institute of Epidemiology (NIHE) in Hanoi conducted further nationwide surveillance around the same period of time as the DOMI study. The average number of typhoid cases in Vietnam across the country in all ages was estimated to be 11,696, corresponding with an average national incidence rate of 14.7/100,000 population per year <sup>363</sup>. During this period (1999 to 2003) two out of the 63 provinces of Vietnam (Soc Trang in the south and Dien Bien in the north) were estimated to have particularly high incidences (>100/100,000 population per year) (Figure 3.2A); a further 18 were estimated to have a medium incidence (>10<100/100,000 population per year). The propensity of the disease was understood to arise in children, with an estimated incidence of 36.6/100,000

population aged <15 years per year <sup>363</sup>. Lastly, in 1998, Lin and others <sup>87</sup> estimated a population incidence of 198/100,000 population in Dong Thap province in the Mekong Delta, equating with a crude incidence of 930/100,00 people (Figure 3.1).



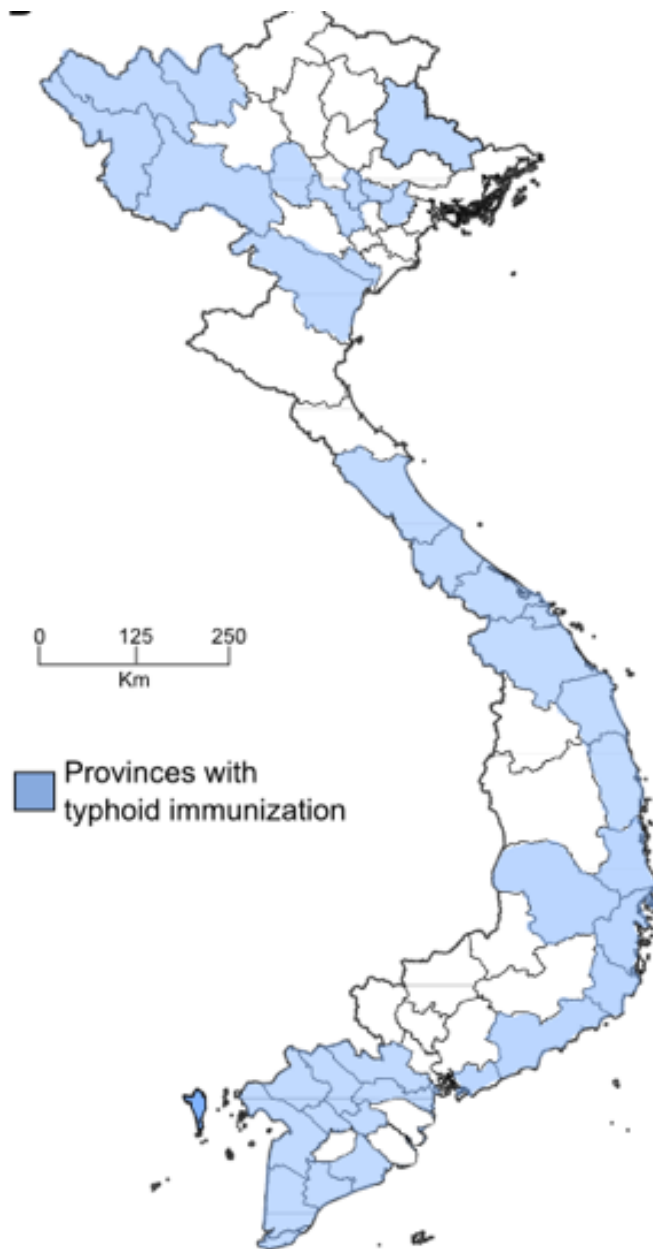
**Figure 3.1** Historic crude estimates of typhoid fever incidence in Vietnam

Histogram showing the estimated crude incidences (on a log scale) of typhoid fever in Vietnam from government records (GR) and subnational incidence estimates available from Ochiai *et al.* (aggregated estimated from 2002-2004 with 2003 as the midpoint from Hue province in people aged 5-18 years) and Lin *et al.* (aggregated estimated from 1997-2000 with 1998 as the midpoint from Dong Thap province) <sup>41,360</sup>.



**Figure 3.2 (A)** Map of Vietnam showing estimated disease incidences and provinces implemented Vi immunization

A) North orientated map of Vietnam showing the estimated incidence of typhoid fever in Vietnam from government data between 1999 and 2003. Provinces with high, medium, low incidence are highlighted by shading (see key).

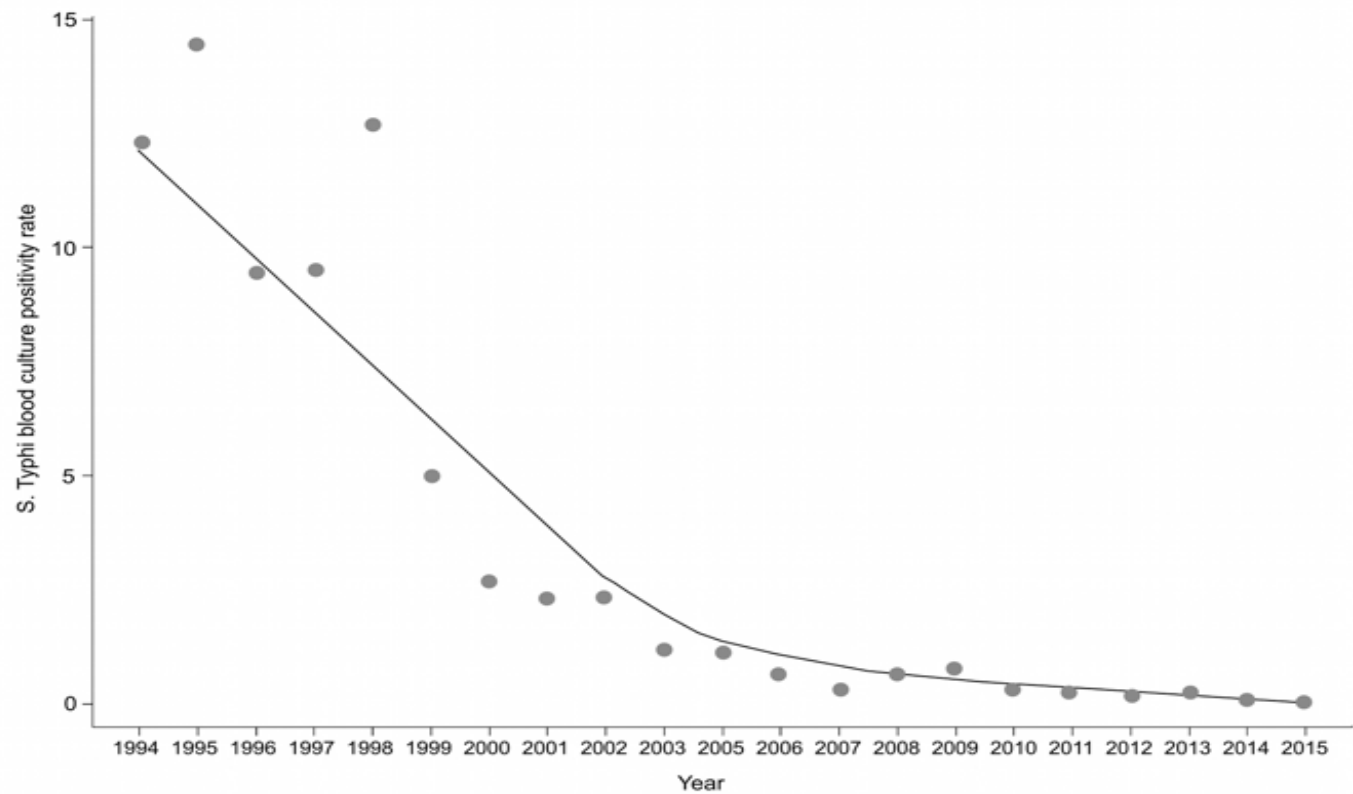


**Figure 3.2 (B)** Map of Vietnam showing estimated disease incidences and provinces implemented Vi immunization

B) North orientated map of Vietnam showing the 35 provinces in Vietnam in 2005 that were incorporated into the national typhoid Vi immunization campaign; blue shading (see key). Maps are reproduced from Cuong N. Typhoid Vaccine Used in Vietnam and its Impact. In: Consultation on Typhoid Vaccine Introduction and Typhoid Surveillance <sup>363</sup>.

### 3.3 Typhoid trends in Ho Chi Minh City

Routine blood culture data from the Hospital for Tropical Diseases (HTD) in Ho Chi Minh City (HCMC) in the south of Vietnam between 1994 and 2014 highlights a major reduction in the prevalence (and absolute number) of positive blood cultures for *S. Typhi* over time (Figure 3.3)<sup>81</sup>. HTD is a sentinel infectious disease hospital that serves as a primary and secondary facility for the surrounding local population in HCMC and a tertiary referral centre for 17 provinces in the south of the country, and, therefore, has catchment population of approximately 40 million people. The highest rates of positive blood cultures for *S. Typhi* at HTD were recorded in 1995 and 1998, when the proportion of positive blood cultures for *S. Typhi* was 14.5% and 12.8% (of all blood cultures taken), respectively. In the late 1990s this figure began to show an annual decline; 5% *S. Typhi* blood culture positivity rate of all blood cultures taken in 1999. After the turn of the millennium the number of culture positive cases of typhoid fever at HTD continued to decrease annually, with the prevalence of *S. Typhi*-positive blood cultures not rising higher than 1 per cent from 2005 onwards. Therefore, in the absence of contemporary (and accurate incidence) data, if we extrapolate these trends we can surmise that presently the incidence of enteric fever in Vietnam is probably exceptionally low (<10/100,000 population per year), and there has been a remarkable and sustained decline in the prevalence of *S. Typhi*-positive blood cultures in HTD and other health care facilities across the country.



Total cultures	4045	4947	3775	3640	4694	3438	3596	3444	3878	3985	4554	4764	6529	6325	4497	7543	8777	8781	9774	10346	9631
S. Typhi positive	497	716	357	346	599	171	96	80	91	49	52	30	20	40	34	24	21	14	20	9	7

**Figure 3.3** The decline in *Salmonella* Typhi positive blood cultures in a sentinel infectious disease hospital in Ho Chi Minh City

Plot showing the proportion of total blood cultures taken from which *Salmonella* Typhi was isolated between 1993 and 2015 at the Hospital for Tropical Diseases in Ho Chi Minh City, with a LOESS curve. The total number of blood cultures taken and the number from which *Salmonella* Typhi were isolated are shown at the base of the figure.



In a pattern similar to those observed in parts of sub-Saharan Africa (but not in the same magnitude), there has been a replacement of “classical” community-acquired pathogens in bloodstream infections (such as *S. Typhi*) with those more commonly associated with HIV infection and the current international epidemic of AMR bacteria <sup>364</sup>. Specifically, assorted fungal pathogens, multi-drug resistant (MDR) non-*Salmonella* Gram-negative bacteria, and non-typhoidal *Salmonella* (NTS) now dominate the bloodstream infection landscape in Vietnam <sup>81,365,366</sup>. Paratyphoid fever, which is associated with the various pathovars of *Salmonella* Paratyphi (*S. Paratyphi* A, B, and C), has been reported to be increasing in prevalence in parts of Asia <sup>367</sup>. This surge is specifically associated with *S. Paratyphi* A; however, the isolation of this organism is rare in Vietnam (and across Southeast Asia) and is generally limited to extended sporadic outbreaks, as recently observed in neighbouring Cambodia <sup>368</sup>. A study conducted at HTD cultured less than 7 *S. Paratyphi* A isolates per year between 1998 and 2008, this subsequently declined to zero from 2008 <sup>81</sup>. However, in 1990, Global Burden of Disease estimated incidence of paratyphoid to be 81/100,000 population; this was estimated to be 40/100,000 in 2016 <sup>70</sup>.

### **3.4 Antimicrobial susceptibility**

Traditionally, Vietnam has been a global hotspot for MDR *S. Typhi*, which is defined as resistance against the first-line antimicrobials, ampicillin, chloramphenicol, and trimethoprim-sulphamethoxazole <sup>369</sup>. These latter days, first-line regimes were commonly prescribed in the community and in health care settings for the treatment of typhoid fever, and many non-specific febrile diseases, in Vietnam in the 1980s and early 1990s. The first notable spike of MDR *S. Typhi* in

Vietnam arose in the early 1990's, correlating with a major peak in *S. Typhi*-positive blood cultures at HTD in HCMC <sup>370</sup>. This MDR phenotype in *S. Typhi* was associated with an *incH1* plasmid backbone <sup>103</sup>, which has been consistently identified within *S. Typhi* isolated in Vietnam and coupled with organisms belonging to a specific phylogenetic group known as haplotype 58 (H58) <sup>371</sup>, now designated genotype 4.3.1 <sup>372</sup>. These organisms, with this same MDR phenotype, were described as still circulating in high numbers in the Mekong Delta region, some 150 km away from HCMC, in 2004 and 2005 <sup>373</sup>.

*Salmonella enterica* serovar Typhi belonging to genotype 4.3.1 are also commonly associated with a mutation (S83F) in the DNA gyrase gene *gyrA*, catalysing resistance against nalidixic acid and reduced susceptibility against the second-generation fluoroquinolones, ciprofloxacin, and ofloxacin <sup>374,375</sup>. Variants with this specific *gyrA* mutation began to emerge in Vietnam in the early 1990s <sup>376</sup>, shortly after the introduction of quinolones for the treatment of non-specific febrile diseases when the first-line treatments became less effective at inducing defervescence. The secondary peak in *S. Typhi* cases in routine blood culture data from HTD was associated with the emergence of organisms exhibiting resistance against nalidixic acid and reduced susceptibility against fluoroquinolones <sup>81,377</sup>. These organisms, specifically genotype 4.3.1 *S. Typhi* with an S83F mutation in *gyrA*, have since become the most prevalent variant in Vietnam <sup>373</sup>, reflecting the pattern of the molecular epidemiology of *S. Typhi* across much of Asia <sup>375</sup>. Current information regarding the AMR profiles of the extant *S. Typhi* population in Vietnam are limited, but our unpublished data suggests that MDR strains have all but disappeared in the southern part of the country and *S. Typhi* with an S83F and

reduced susceptibility to fluoroquinolones continue to circulate. Notably, despite the sustained use and availability of fluoroquinolones in Vietnam for various bacterial infections there has not yet been the emergence of *S. Typhi* exhibiting resistance against ciprofloxacin and ofloxacin, as has been observed elsewhere in Asia <sup>378</sup>.

### **3.5 Vaccination campaigns**

The Vietnamese government recognized the public health issue of typhoid fever in the 1990s and initiated several vaccine campaigns with internationally manufactured vaccines in an attempt to protect the most vulnerable groups within the population, most commonly children <sup>379</sup>. Further, NIHE in Hanoi was pioneering in instigating locally manufactured Vi Polysaccharide vaccine and distributing it as control measure through the public health network. However, despite a Vi conjugate vaccine and a new oral attenuated typhoid vaccine being trialled in Vietnam for the first time these were never introduced as public health interventions <sup>319,380</sup>. All the national typhoid Vi vaccine programs conducted between 1997 and 2012 were executed as school-based campaigns, immunizing children between the ages of three and 10 years. Between 1997 and 2003 the Vietnamese government immunized more than 4,000,000 children aged three to five years with TYPHIM Vi polysaccharide, manufactured by Aventis Pasteur. After manufacturing their own Vi polysaccharide vaccine through the Institute of Vaccines and Medical Biologicals/DaLat Pasteur Vaccine Company (IVAC/DAVAC), the Vietnamese public health system administered more than 2,000,000 additional doses to children aged five to ten years (2004-2010), and latterly children aged three to five years (2011-2012). At the peak of these Vi vaccine campaigns in 2005 (1,200,000 doses), children were being immunized in 35 different provinces across the country (Figure 3.2B) <sup>363</sup>. The

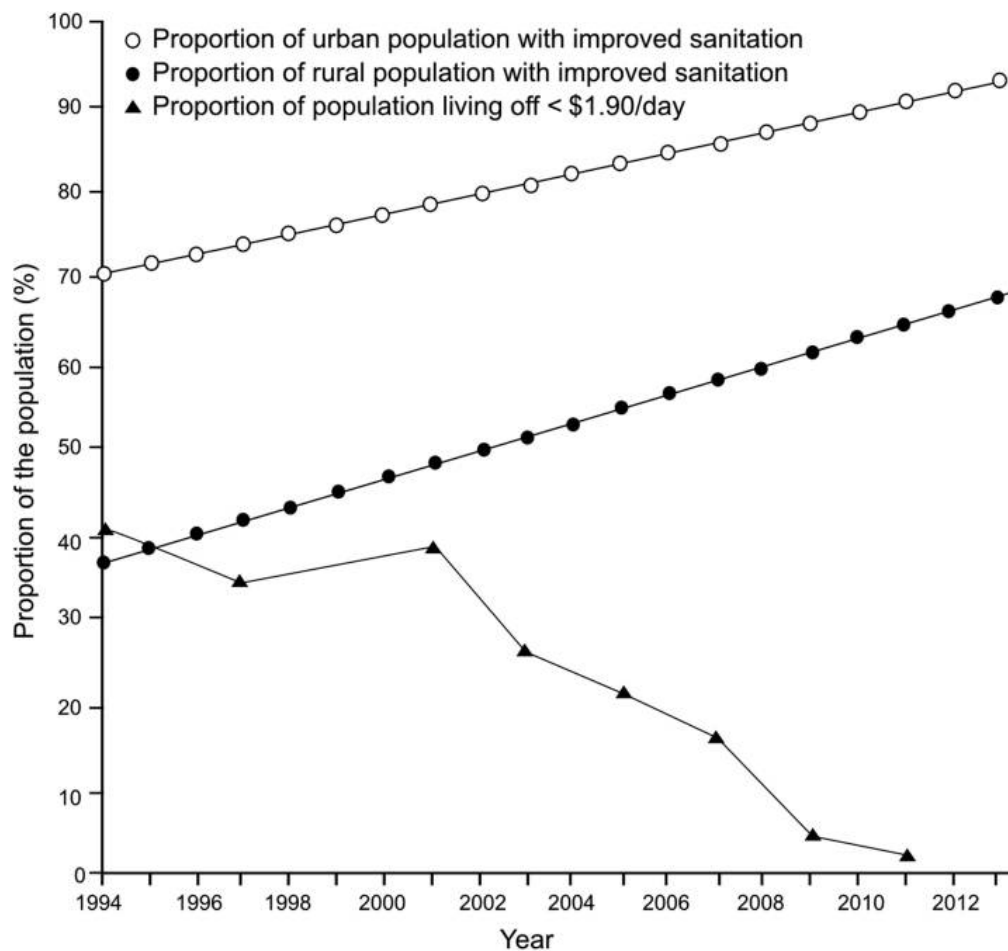
coverage rate of these campaigns was high, with greater than 90 per cent of the target population receiving a Vi vaccine between 1999 and 2010. Population based data assessing the direct effect (i.e. without additional sanitation covariates) of these immunization programmes on typhoid incidence are unavailable, but a there was a substantial decrease in the incidence of typhoid fever across the country between 1997 and 2007, most notably in the northwest of the country and Mekong River Delta in the South <sup>363</sup>, which were covered extensively by the immunization program (Figure 3.2) <sup>363</sup>.

### **3.6 Contextual factors that may have influenced typhoid fever incidence**

Being of low socioeconomic status is a major risk factor for contracting typhoid fever <sup>337</sup>. Vietnam has been through an unprecedented period of economic development since the mid 1990s, which has had a substantial knock-on effect on the reduction of poverty and poverty-associated communicable diseases <sup>381</sup>.

Between 1998 and 2014 Vietnam's Gross Domestic Product (GDP) increased from \$27 billion to \$86 billion, and the Gross National Income (GNI) per capita increased from \$360 to \$1,890 over the same time period <sup>382</sup>. These figures correlate with a reduction in national poverty (poverty headcount ratio at \$1.90 a day declining from 34.8 percent in 1998 to 3.2 per cent in 2012) (Figure 3.4) and a waning in typhoid fever incidence, but overlap with the period of the national immunization campaigns. In addition, multiple contextual factors are considered to have had an effect on the incidence of typhoid fever. Typhoid fever is associated with poor water quality and evidence of the organism in water supplies can be measured using molecular methods <sup>383</sup>. Consequently, water, sanitation, and hygiene (WASH) conditions are one of the major factors in assessing disease control. Sub-national

data on water and sanitation extracted from Multiple Indicator Cluster Surveys (MICS) in Vietnam show that the fraction of the population in the southeast of Vietnam with access to improved sanitation facilities (means of excreta disposal that decrease human contact with faeces) increased from only 42% in 1995, to 93.6% in 2014 <sup>384</sup>. Furthermore, the proportion of the south-eastern population of Vietnam with improved water sources (as per MICS standards; water piped into homes or yard, public taps, standpipes, protected wells, or springs) rose from 93.6% in 2006 to a peak of 98.4% in 2011 <sup>384</sup>. Correspondingly, data from the World Bank shows that there was a steady increase in the proportion of the Vietnamese population with access to improved sources of drinking water between 1998 (74.5 per cent of the population) and 2014 (96.4 per cent of the population) <sup>385</sup>. Similarly, there was an increase in the proportion of the population with access to improved sanitation facilities, including flush and slab latrines, over the same period; 49.5 per cent of the population in 1998 to 76.3 per cent of the population in 2012 <sup>385</sup>.



**Figure 3.4** The reduction of poverty and improvements in sanitation in Vietnam  
(data from World Bank <sup>385</sup>)

Plots showing; the proportion of the Vietnamese population living on <\$1.90 a day (black triangles), the proportion of the Vietnamese rural population with improved sanitation (black circles), and the proportion of the Vietnamese urban population with improved sanitation (white circles) from 1994 to 2013.

### 3.7 Knowledge gaps

All available data suggests that the trend of typhoid fever incidence began to exhibit a steep decline in Vietnam from 1999 onwards. Therefore, the outstanding questions regarding the dramatic reduction in typhoid fever in Vietnam are: to what extent did immunization play a role in reducing typhoid fever? and how much influence did economic growth have on improving living standards to reduce all waterborne diseases, including typhoid fever? Given the paucity of data regarding the longitudinal incidences of typhoid fever (and other waterborne diseases) in Vietnam, disaggregating the independent effect of these differing approaches is a major challenge. Furthermore, dissimilar demographics, disease epidemiology, and disease incidence across the provinces of Vietnam make it impractical to assess the overall impact of a specific intervention in any given location. The data that are available are inconclusive and limited by disease time trends from a single tertiary referral hospital in HCMC. As a result, these data do not provide insight into regional differences in typhoid fever reduction across Vietnam or characterize the current national burden. Furthermore, there is no systematic data regarding intestinal perforation (although this has been described in the southern provinces of Vietnam<sup>386</sup>), other severe disease presentations<sup>23</sup>, or typhoid associated mortality. As a result, it is impossible to directly assess any trends associated with these severe outcomes.

Regardless of the precise role of any single intervention, the reduction and virtual elimination of typhoid fever in Vietnam, largely driven by strong political will, as evidenced by sanitation improvements, better health care, and immunization campaigns, should be considered a major public health success. However, this

success story does not quite replicate into a blueprint in how typhoid fever should be controlled across Asia, that is, what worked in Vietnam may not work in precisely the same way in other locations. It is likely that the various vaccination campaigns that have taken place in Vietnam over the last 15 years have contributed to the observed decreasing trend in typhoid fever, but their effect is difficult to assess. However, the immunization campaigns were conducted in children in selected provinces, and these individuals received a single dose of the Vi polysaccharide vaccine. This vaccine only provides limited efficacy in the first year after immunization and there is a rapid decline in antibody titre two years after receiving the vaccine<sup>387,388</sup>; herd protection remains variable. Therefore, I conclude that economic development, improved access to clean water supplies, better sanitation, and a reduction in poverty probably played the greatest combined role in reducing the incidence of typhoid in Vietnam. Quantifying the precise contribution of these in reduction of typhoid is again problematic, and there are multiple additional factors that should also be considered that may confound these interactions. It is, however, worth stating that *S. Typhi* appears to be profoundly sensitive to improvements in sanitation, which reduces human exposure to the organism, thus, lessening disease incidence and person-to-person transmission.

### **3.8 Conclusions**

The reduction of typhoid fever in Vietnam has been remarkable, and has been largely driven by economic development and improved living standards for the population. Immunization has probably had some impact on disease reduction, but the use of an imperfect vaccine may only provide limited respite in disease transmission without required improvements in WASH. Better-designed WASH



intervention studies with disease endpoints and systematic incidence data are required to glean a greater understanding of the precise contextual factors that impact on typhoid fever incidence.

## **Chapter 4 An evaluation of purified *Salmonella* Typhi protein antigens for the serological diagnosis of acute typhoid fever**

### **4.1 Introduction**

Typhoid occurs only in humans, making it a disease that can technically be eradicated <sup>182</sup>. Indeed, typhoid has all but been eliminated from several countries in Southeast Asia where it was the most common cause of hospitalized febrile disease 20-30 years ago <sup>81,287</sup>. Elimination in these areas is generally attributed to extensive improvements in sanitation rather than widespread immunization schemes. The lack of data regarding the long-term impact of mass immunization for typhoid and the performance of licensed vaccines have hindered immunization as a sustainable typhoid control and elimination strategy. Future considerations for rational control measures for typhoid will rely on more accurately assessing disease burden, which requires a reliable diagnostic approach <sup>389</sup>.

All commonly used typhoid diagnostics perform poorly and are a roadblock for disease control efforts <sup>390,391</sup>. Currently, the only reliable method for the identification of febrile individuals with typhoid is the culture of a causative organism from a biological specimen <sup>391,392</sup>. However, this procedure is restricted to laboratories with adequate equipment and microbiology training, and the method has a limited sensitivity due to low concentrations of organisms in the peripheral circulation <sup>281,393,394</sup>. Low bacterial loads have a similar impact on other methods that rely on detecting the presence of the infecting organisms, such as antigen detection or nucleic acid amplification. These methods are often reported to be highly sensitive, but have unrealistic performances; pre-treatment with

antimicrobials is likely to compound this issue further <sup>281,390</sup>. New typhoid diagnostics are a necessity and various approaches have been evaluated, including measurement of innate immune responses <sup>164,395</sup>, antibody in lymphocyte supernatants <sup>222,396</sup>, and the identification of metabolomic signatures <sup>397</sup>. However, these advances are still restricted to research laboratories and are not yet ready to be developed into simple, rapid diagnostic tests (RDTs).

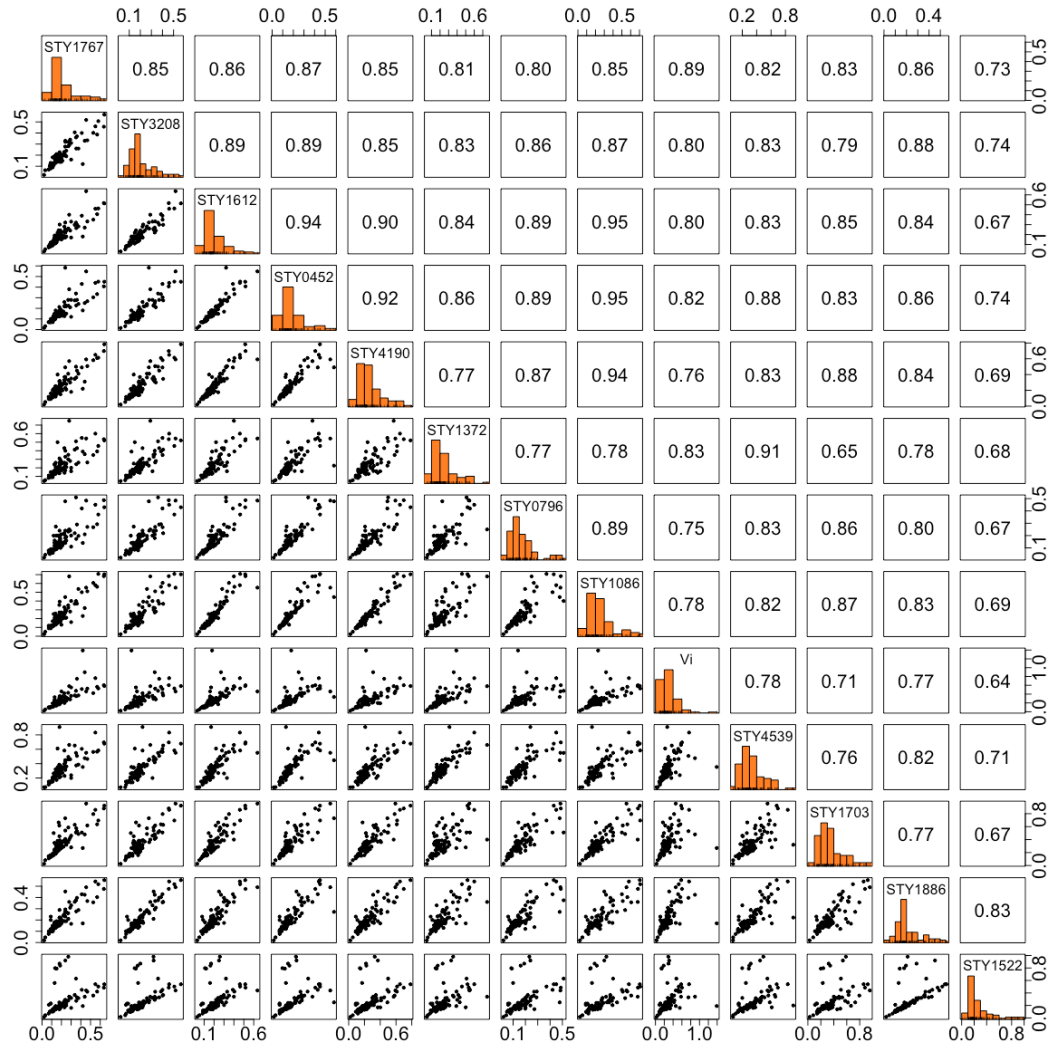
I previously exploited a protein microarray to identify a multitude of immunogenic *S. Typhi* protein antigens to which an antibody response was generated during the early stages of typhoid <sup>340</sup>. With the aim of validating antigens that could be used in a diagnostic assay I expressed and purified several of these potentially serodiagnostic *S. Typhi* antigens and investigated their diagnostic performance in a cohort of febrile Bangladeshi patients.

## **4.2 Results**

### **4.2.1 Acute IgM antibody responses against *Salmonella Typhi* antigens**

Of the 18 protein antigens targeted, 12 of them were successfully purified (Table 2.2). ELISAs were independently performed employing the purified protein antigens and the Vi polysaccharide to detect IgM in the plasma of 40 afebrile control subjects, 17 febrile individuals with a confirmed infection other than typhoid, and 32 individuals with either blood culture or PCR (or both) confirmed typhoid infections (n=89 samples). IgM against all twelve of the purified *S. Typhi* proteins and the Vi polysaccharide were measured in all of the 89 samples subjected to ELISA. IgM titres from each of the antigens individually were compared to assess the performance of the antigens and to identify potential correlations between the

serological targets. I found that early IgM responses against the majority of the novel *S. Typhi* protein antigens, with the exception of STY1522 ( $\rho < 0.7$ ,  $p < 0.05$ , Spearman's correlation coefficient test), were highly correlated with one other ( $\rho > 0.8$ ). Notably, the correlations between IgM response to the protein antigens and to the Vi polysaccharide were relatively weaker ( $\rho < 0.8$ ) than those between two protein antigens (Figure 4.1).



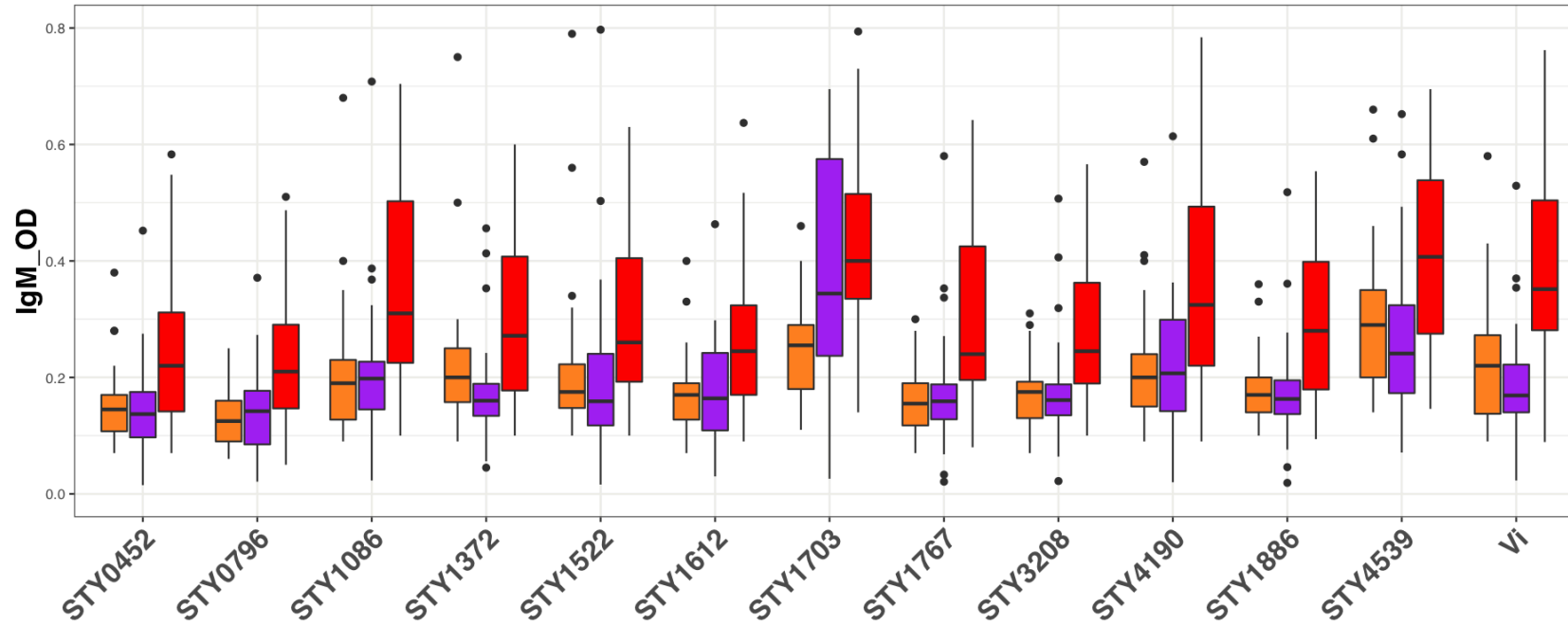
**Figure 4.1** Spearman correlation among antigen-induced antibodies

The names of the various antigens and histograms showing the distribution of IgM antibody against these antigens are presented on the diagonal. Scatterplots below the diagonal represent the correlation of IgM measurements of the two antigens on a right angle to the plots. The numerals above the diagonal depict the Spearman correlation coefficient ( $\rho$ ) values of the mirrored plots. OD values of each antigen-induced antibody are presented to the side of the corresponding scatterplots.

#### 4.2.2 The diagnostic potential of IgM against *Salmonella* Typhi antigens

IgM against all twelve of the protein antigens and the Vi polysaccharide was significantly elevated in the plasma of the typhoid patients in comparison with the afebrile controls ( $p < 0.05$ , Wilcoxon signed-rank test) (Figure 4.2). Furthermore, there was a significant differentiation in the plasma IgM measurements between the typhoid patients and those with a febrile disease with an alternative confirmed aetiology with all antigens ( $p < 0.05$ , Wilcoxon signed-rank test) with the exception of STY1522.

By assessing raw antibody measurements, I surmised that the best performing antigens, with respect to differentiating between the patient groups were STY4539, STY1886, and Vi (Figure 4.2). The mean IgM responses (OD values) in the afebrile controls, the other confirmed infections and the typhoid infections were 0.29, 0.27, and 0.42 (against STY4539), 0.17, 0.18, and 0.25 (against STY1886), and 0.22, 0.21, and 0.35 (against Vi), respectively. This segregation between the patient groups was highly significant, resulting in  $p$ -values of 0.0001 and 0.003 for IgM against STY4539;  $< 0.0001$  and 0.004 for IgM against STY1886; and 0.0001 and 0.0001 for IgM against Vi, between afebrile controls and typhoid infections and between other febrile diseases and typhoid infections, respectively (Wilcoxon signed-rank test).



**Figure 4.2** IgM responses against *Salmonella* Typhi antigens in a Bangladeshi cohort of febrile patients and controls

Boxplots showing IgM measurements (optical density) in plasma from afebrile controls (orange), febrile patients with an infection other than typhoid fever (purple), and confirmed typhoid patients (red). Dark horizontal lines represent the mean IgM measurement, with the box representing the 25th and 75th percentiles, whiskers represent the 5th and 95th percentiles; outliers are represented by dots. A) Boxplots of antibody responses against (from left to right) STY0452, STY0796, STY1086, STY1372, STY1522, STY1612, STY1703, STY1767, STY3208, STY4190, STY1886, STY4539 and Vi. All mean antibody measurements were statistically significant between the afebrile controls and typhoid infections and between other infections and typhoid infections ( $p < 0.05$  Wilcoxon signed rank test), with the exception of STY1522.

#### **4.2.3 Sensitivity and specificity of the serodiagnostic antigens**

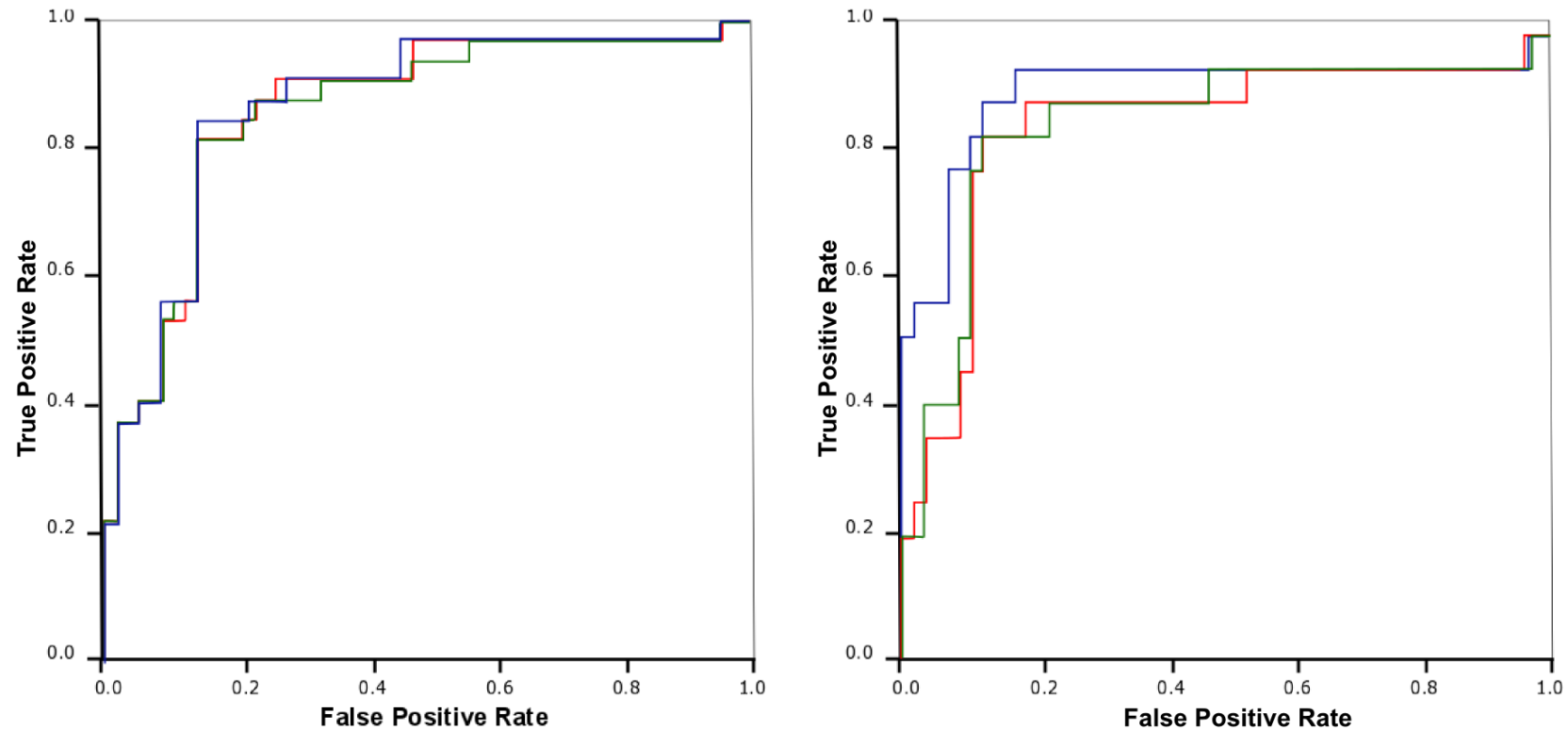
To further assess the IgM responses against the various *S. Typhi* antigens for the purposes of diagnostic sensitivities and specificities were calculated using a validation group incorporating two positive reference groups and a negative reference group for additional statistical power. The negative reference group (n=57) was the combination of data from the afebrile controls (n=40) and from those with a confirmed diagnosis other than typhoid (n=17 cases). The assay results were validated independently with two sets of positive reference data; these were a combination of blood culture confirmed *S. Typhi* along with those with a positive PCR amplification result for *S. Typhi* from blood (n=32), and the blood culture confirmed *S. Typhi* patients (n=19).

The IgM responses against each of the antigens generated a continuous data set that was used to generate ROC curves to optimize the index cut-off value. The defined cut-off values of the thirteen antigens corresponded with a range of sensitivities ranging from 0.50 to 0.84 and specificities between 0.58 and 0.84; areas under the ROC curve (AUC) ranged from 0.7 to 0.85. When used alone, none of the antigens demonstrated a sensitivity or specificity >0.8. As predicted, Vi, STY4359, and STY1886 were the three antigens with the greatest serodiagnostic capacity in discriminating typhoid cases from afebrile controls and other infections. The sensitivities and specificities for identifying typhoid patients by IgM titres were 0.68, 0.8 (Vi); 0.62: 0.82 (STY4539); and 0.62; 0.82 (STY1886) respectively. Correspondingly, the AUCs were 0.84 (95%CI: 0.71, 0.96), 0.77 (95%CI: 0.61, 0.92), and 0.77 (95%CI: 0.62, 0.91).



I next employed SVM to identify combinations of two or more antigens across all 13 antigens to increase overall sensitivity and specificity. Using confirmed *S. Typhi* infection by blood culture or PCR as the positive reference (n=32), I found 11 combinations of two to four antigens that gave sensitivities from 0.81 to 0.84, specificities from 0.81 to 0.86, and AUCs from 0.87 to 0.89 (Figure 4.3). I additionally identified 17 combinations of two to four antigens when using the positive reference as culture confirmed *S. Typhi* only (n=19), obtaining sensitivities from 0.84 to 0.89, specificities from 0.88 to 0.94, and AUCs from 0.86 to 0.91 (Figure 4.3).

For the positive reference sets, IgM against Vi contributed to all of the combinations, while STY1703, STY1886, and STY4539 were present in more than half of the combinations. The remaining nine antigens contributed to at least one combination that gave sensitivities and specificities >0.8. These results demonstrated that, in the majority of examples, a combination of up to four antigens was directly associated with an increased performance of the IgM serology. However, the best performing antigens for the identification of typhoid patients by IgM were Vi in combination with either STY1703 or STY1886. Some representative combinations are presented in Table 4.1 and Table 4.2.



**Figure 4.3** Assessing the sensitivity and specificity of IgM against *Salmonella* Typhi antigens for the diagnosis of typhoid fever

Receiver operating characteristic (ROC) curves summarizing the antibody responses against antigen combinations for the diagnosis of typhoid. The x-axis displays the false positivity rate (1-Specificity) and the y-axis displays true positive rate (Sensitivity). The performance of two, three and four antigens are shown by the red, green, and blue lines, respectively. A) ROC curve produced when the positive references are typhoid cases confirmed by blood culture and PCR amplification (n=32). B) ROC curve produced when the positive references are typhoid cases confirmed by blood culture only (n= 19).

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**Table 4.1** The sensitivity and specificity of using multiple antigens for typhoid diagnosis using blood culture and PCR positive patients as positive reference group (n=32)

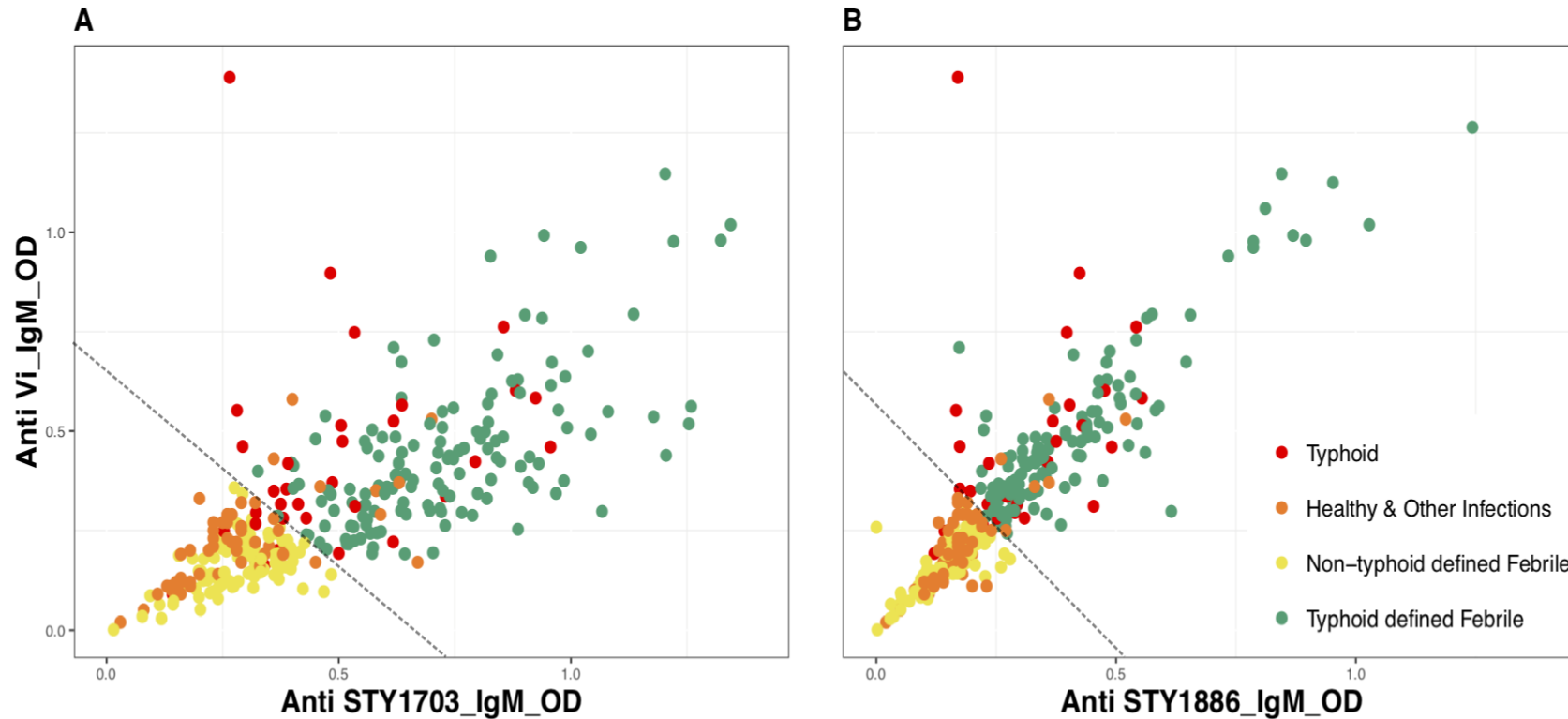
Antigen combinations	STY1703 & Vi	STY4539 & STY1703 & Vi	STY4539 & STY1703 & STY1886 & Vi
Specificity	0.86	0.86	0.86
Sensitivity	0.81	0.84	0.84
Number positive with undiagnosed febrile disease (n=226)	135 (59%)	134 (59%)	134 (59%)
Number positive with clinically suspected typhoid (n=18)	16 (88%)	16 (88%)	16 (88%)
AUC (95%CI)	0.865 (0.782, 0.947)	0.863 (0.779, 0.947)	0.866 (0.783, 0.949)

**Table 4.2** The sensitivity and specificity of using multiple antigens for typhoid diagnosis using blood culture positive patients only as positive reference group (n=19)

Antigen combinations	STY1886 & Vi	STY4190 & STY1886 & Vi	STY4539 & STY4190 & STY1886 & Vi
Specificity	0.88	0.89	0.88
Sensitivity	0.84	0.84	0.89
Number positive with undiagnosed febrile disease (n=226)	119 (52%)	78 (34%)	71 (31%)
Number positive with clinically suspected typhoid (n=18)	15 (83%)	13 (72%)	13 (72%)
AUC (95%CI)	0.859 (0.746, 0.972)	0.891 (0.791, 0.991)	0.912 (0.81, 1.014)

#### **4.2.4 Identifying typhoid cases in patients with undiagnosed febrile disease**

In this Bangladeshi cohort there were 226 patients with febrile disease without laboratory confirmed aetiology and 18 with clinically suspected typhoid that were negative by blood culture and PCR amplification. I aimed to estimate the proportion of patients in this population who may have typhoid by applying the SVM cut-offs and combining the IgM titres against two *S. Typhi* antigens. I performed two independent analyses; the first combined IgM titres against STY1703 and Vi using a combination of culture confirmed *S. Typhi* and positive PCR amplification for *S. Typhi* from blood as the positive reference group (Figure 4.4 and Table 4.2). Using these criteria, I found that 135/226 (59%) of the undiagnosed febrile patient group and 16/18 (88%) with clinically suspected typhoid had IgM titres indicative of typhoid. Using more stringent criteria (blood culture confirmed patients) as the positive reference and a combination of IgM against Vi and STY1886 I found that 119/226 (52%) febrile cases and 15/18 (83%) clinically suspected typhoid, respectively, had a profile indicative of typhoid (Figure 4.4).



**Figure 4.4** Detecting febrile patients with an IgM profile indicative of typhoid fever

Hyperplane plots predicting the number of undiagnosed febrile patients that have an IgM measurement indicative of typhoid fever. The orange points represent the negative controls, which includes afebrile controls and patients with other infections. The red points represent typhoid cases confirmed by blood culture or PCR. Yellow points are febrile patients with an IgM profile indicative of not having typhoid fever, whilst the green points are febrile patients defined as having a typhoid infection using pre-defined hyperplane IgM profile. A) Plot where hyperplane was defined using the positive reference as the typhoid cases confirmed by blood culture or PCR (n=32); the selected combination of antigens was STY1703 and Vi. B) Plot where hyperplane was defined using the positive reference as the typhoid cases confirmed by blood culture only (n=19); the selected combination of antigens was STY1886 and Vi.

### 4.3 Discussion

Typhoid is caused by a human restricted pathogen that is transmitted faecal-orally. Consequently, improving the quality of drinking water supplies and education in better hygiene practices are likely the most effective measures for typhoid elimination. However, these interventions cannot be promptly realized in the endemic areas of Africa and South Asia. Therefore, the short-term control of typhoid is dependent on large vaccination programmes and appropriate treatment, both of which, for differing reasons, rely on better case detection. The fact that there is not currently a typhoid diagnostic assay with a high degree of sensitivity or specificity limits disease burden assessments<sup>80,398</sup>, and may result in patients being misdiagnosed and receiving sub-optimal therapy<sup>399</sup>. Furthermore, with the global increase in *S. Typhi* associated with reduced susceptibility and resistance against the fluoroquinolones and other antimicrobials<sup>378,400</sup>, the demand for typhoid diagnostics are now greater than ever<sup>401,402</sup>.

There are a paucity of data arising from studies on humans with typhoid that have measured the immunological response to *S. Typhi* specific antigens<sup>182</sup>. Studies that have been performed have found significant interferon- $\gamma$  (IFN- $\gamma$ ) responses in cells stimulated with various antigens including fimbriae and outer membrane proteins using polymorphonuclear cells from the blood of typhoid patients<sup>403</sup>. Further, when whole blood from typhoid patients is stimulated with *S. Typhi* lipopolysaccharide, tumour necrosis factor  $\alpha$  (TNF- $\alpha$ ) release is lower during active typhoid than after antimicrobial treatment, indicating a short immune modulation effect, which may be induced by the Vi polysaccharide<sup>404</sup>. Antigen arrays and other probing techniques have been used successfully to interrogate the antibody repertoire during early

infection <sup>340,405–407</sup>, and have detected antibody responses to several novel, and potentially organism specific, antigens that may be able to distinguish typhoid patients from controls <sup>340</sup>. Using this “screening data” I rationally selected several *S. Typhi* protein antigens and aimed to investigate if early immunological diagnostic signals could be detected in the plasma of febrile patients.

My study focused on a well-defined patient group from Bangladesh, who were enrolled for the primary focus of studying typhoid diagnostics. Whilst the patient numbers with typhoid in this group were relatively modest, the clinical and laboratory criteria for patients with febrile disease were consistent and have been previously assessed with commercial serological tests for typhoid <sup>335</sup>. I noted that the IgM response against the twelve purified *S. Typhi* protein antigens were stable and well correlated; the IgM responses in comparison to Vi correlated less consistently. These data confirm my original antigen screening data and suggest that these antigens are immunogenic and induce an antibody response early in infection. Given the poor performance of commercial RDTs <sup>267,408</sup>, my data signify that the early detection of IgM against more specific *S. Typhi* protein antigens may be a more specific and sensitive approach for developing a RDT for typhoid.

Indistinguishable clinical features and the lack of a reliable gold standard test complicate typhoid diagnosis. Here, the IgM response against all 12 antigens was significantly higher in typhoid patients than both afebrile controls and patients with febrile diseases other than typhoid. Furthermore, through inference from the AUC under the ROC curve I was able to identify the best three performing antigens, which were encoded by STY4539 (PilL) and STY1886 (CdtB) in combination with



the Vi polysaccharide. PilL is a putative exported protein and a component of the type IV pili encoded adjacent to the genes encoding Vi on SPI-7<sup>409,410</sup>. The PilL protein is induced following uptake by human derived macrophages<sup>411</sup>, and the type IV pili to which it is associated facilitates entry into epithelial cells<sup>412</sup>. CdtB, encoded by STY1886, is one of the two A sub-units of typhoid toxin, an AB type toxin<sup>137</sup>. Typhoid toxin is a virulence-associated factor of *S. Typhi*, which is thought to be associated with the early symptoms of typhoid<sup>139</sup>. I confirm that this component of typhoid toxin is immunogenic and may be an important biomarker of acute typhoid<sup>340,413</sup>. Whilst these three virulence factors (PilL, CdtB, and Vi) were not sufficient in themselves to produce a reproducibly high (>0.8) degree of sensitivity and specificity for typhoid diagnosis, I gained additional power by combining data from >1 antigen using an SVM model. The IgM responses against Vi in combination with either PilL or CdtB were found to generate the highest degree of sensitivity and specificity. Seemingly, a combination of the differing IgM responses against polysaccharide and a protein compensates for a lower affinity to one of the antigens. Furthermore, I was able to estimate the proportion of the population that may have typhoid by imposing cut-offs from the typhoid confirmed patients onto the population with undiagnosed febrile diseases. These data did not generate a precise cut-off; therefore, my data suggest that typhoid diagnostics are not an exact science and my data should be interpreted with caution. These methods warrant further investigation in additional cohorts, but it suggests a substantial burden of undiagnosed febrile disease is associated with *S. Typhi* in this setting.

This study has limitations. The sample size was relatively small; I aimed to rectify this by including a subset of patients that appear to have *S. Typhi* DNA in their

bloodstream but were culture negative <sup>89</sup>. Using this combination of methods as a gold standard I was able to increase the diagnostic power of the assays. A further limitation is that this study was conducted in a single healthcare location over a limited time period. Whilst my data provide some confidence that these serological assays may be of utility for typhoid diagnostics, these methods should to be validated in additional cohorts. However, there remains a challenge in identifying typhoid patients that have a sterile blood culture; a combination of novel approaches, such as metabolomics and/or functional genomics <sup>395,397</sup>, in a febrile disease cohort may add further insight into this important patient group.

In conclusion, I have investigated the serological diagnostic potential of *S. Typhi* protein antigens and the Vi polysaccharide in a group of patients with febrile diseases in Bangladesh. My novel data show that serology may have some utility for typhoid diagnostics and a combination of antigens improves the diagnostic potential. My assays give high levels of sensitivity and specificity, but require further assessment in differing patient populations.

## **Chapter 5 Acute and longitudinal humoral immune responses to typhoid toxin after natural infections with *Salmonella* Typhi and *Salmonella* Paratyphi A**

### **5.1 Introduction**

The discovery of typhoid toxin has added greater insights into the ability of *S. Typhi* and *S. Paratyphi* A to induce typhoid fever in humans <sup>137</sup>. Typhoid toxin has been shown in experimental animals to be important for orchestrating the clinical symptoms associated with acute typhoid <sup>138</sup>. However, the role of typhoid toxin in the pathogenesis of typhoid fever during human infection has not, as yet, been determined. A component of typhoid toxin (CdtB; the active subunit) has been shown to elicit an acute IgM response in collection of plasma collected from a well-defined group of febrile patients in Bangladesh, raising the possibility of using the early immune response against this subunit of typhoid toxin for disease diagnosis

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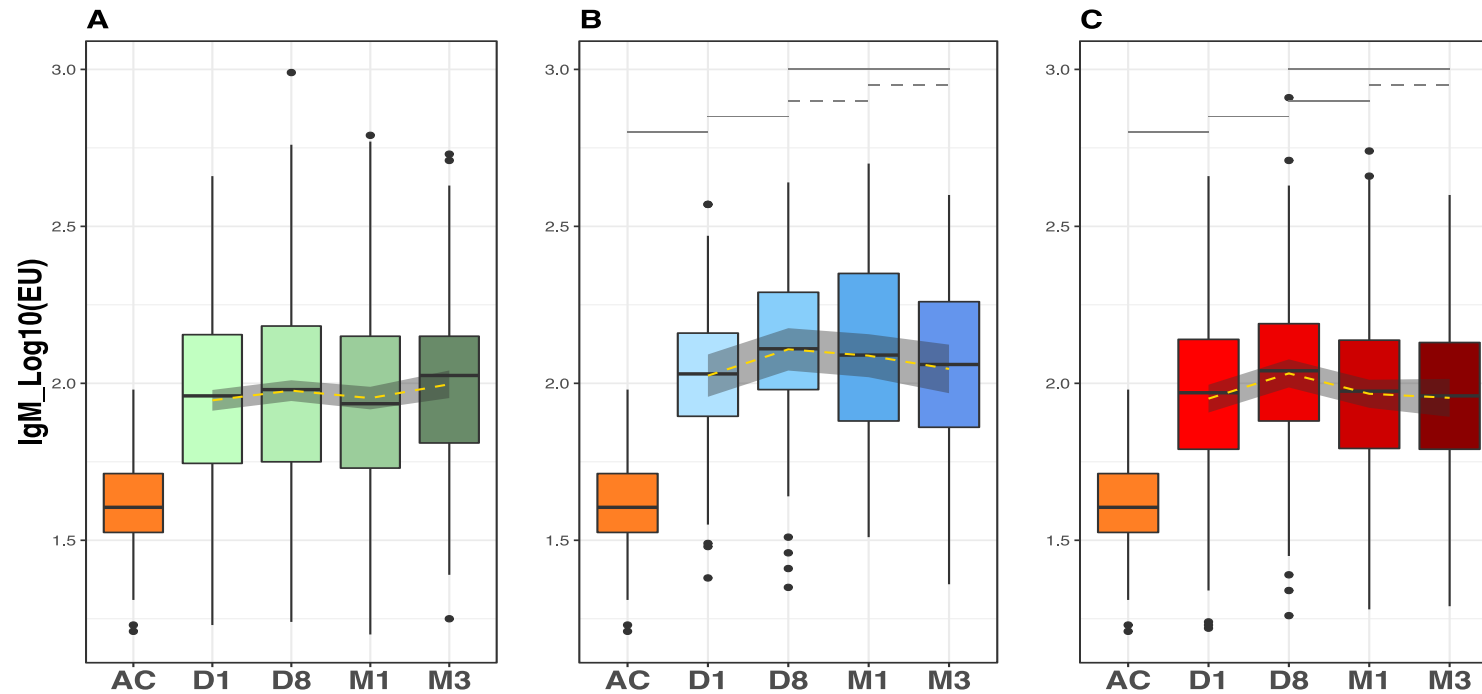
Here, I aimed to measure the natural humoral immune response against typhoid toxin in a group of patients diagnosed with enteric fever to assess the potential of using these responses for diagnosis, and to provide a potential framework for the development of novel therapeutic and prevention strategies. More specifically, I assessed the acute and longitudinal antibody responses against typhoid toxin in a cohort of patients infected with *S. Typhi* and *S. Paratyphi* A in Nepal, and evaluated the toxin neutralizing capacity of acute and convalescent plasma from patients with enteric fever.

## 5.2 Results

### 5.2.1 Longitudinal acute and convalescent anti-toxin IgM responses

ELISAs detecting anti-toxin IgM antibodies (anti-toxin IgM) were performed on a collection of longitudinal plasma samples (up to three-months post presentation) from afebrile community controls (n=49), febrile individuals with an undetermined pathogen (culture negative patients, n=316), and patients with culture confirmed enteric fever (*S. Typhi*, n=152 and *S. Paratyphi A*, n=68) (Figure 5.1).

Overall, these data demonstrated that the concentration of anti-toxin IgM in plasma from community controls was significantly lower than in plasma samples from the three patient groups ( $p < 0.05$ , Wilcoxon signed-rank test). Notably, the concentration of anti-toxin IgM in plasma from the febrile patients with an undetermined pathogen was longitudinally greater than the afebrile community controls; this difference in antibody titres was not significantly different between any of the four sampling points. In *S. Typhi* and *S. Paratyphi A* infected patients, the concentration of anti-toxin IgM in plasma peaked at Day 8 ( $p < 0.05$ , Wilcoxon matched-pairs signed-rank test) and declined steadily until the three-month sampling point. At three months post-infection, anti-toxin IgM titres were significantly lower than those observed on Day 8 ( $p < 0.05$ , Wilcoxon matched-pairs signed-rank test) and were comparable to the baseline measurements recorded on Day 1 ( $p > 0.05$ , Wilcoxon matched-pairs signed-rank test) (Figure 5.1A).



**Figure 5.1** Acute and convalescent IgM response to typhoid toxin.

Boxplots showing the IgM antibody response against toxin (log<sub>10</sub> EU) up to three months after a febrile disease with A) an undetermined pathogen, B) *S. Paratyphi A* and C) *S. Typhi*. Dark horizontal lines represent the mean, with the box representing the 25<sup>th</sup> and 75<sup>th</sup> percentiles, whiskers represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles; outliers are represented by dots. The dashed lines across the boxes represent the trend of the data throughout different time points and the shaded area around trend is 95% confidence interval. The solid/dashed lines above boxes define significantly different distributions ( $p \leq 0.05$ ) or not ( $p > 0.05$ ). AC: afebrile controls, D1: Day 1, D8: Day 8, M1: Month 1, M3: Month 3.

The acute anti-toxin IgM responses from patients infected with *S. Typhi* and *S. Paratyphi A* were indistinguishable. Therefore, as a potential approach for diagnosing enteric fever, I compared the acute anti-toxin IgM profiles from patients with *S. Typhi* and *S. Paratyphi A* infections to those from the febrile patients with an undetermined pathogen. I found that the best compromise between specificity and sensitivity of using anti-toxin IgM titres (alone on day 8 of sampling) for diagnosing enteric fever were 56% and 68%, respectively.

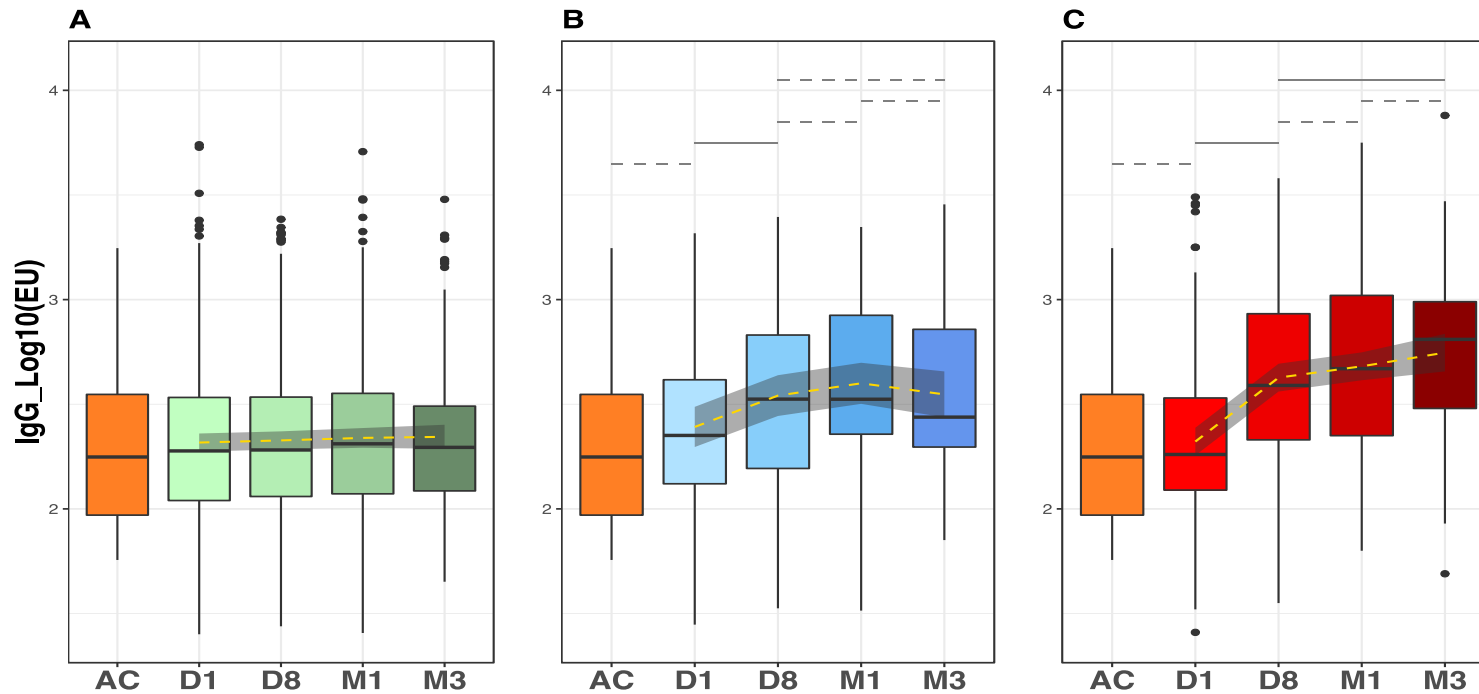
Observing that the specificity and sensitivity were relatively modest, I aimed to improve the classification using additional serological data. I hypothesized that the acute IgM antibody profile against the Vi antigen (anti-Vi IgM) and the O2 antigen (anti-O2 IgM) (the classical surface antigens of *S. Typhi* and *S. Paratyphi A*, respectively) may compensate for the limited performance of anti-toxin IgM to diagnose enteric fever. To test this hypothesis, I additionally measured anti-Vi IgM and anti-O2 IgM in plasma samples from the enteric fever patients and the culture negative patients. The acute antibody profiles against toxin, Vi, and O2 were combined in an attempt to better discriminate culture positive enteric fever cases. The resulting specificity and sensitivity of a combination of anti-toxin IgM, anti-Vi IgM, and anti-O2 IgM titres for diagnosing enteric fever on Day 8 of sampling, in comparison to blood culture using an SVM were 81% and 80%, respectively.

### **5.2.2 Longitudinal acute and convalescent anti-toxin IgG responses**

I next performed ELISAs to detect anti-typhoid toxin IgG antibodies (anti-toxin IgG) in the corresponding collection of patient plasma samples. Overall, the median anti-toxin IgG concentration in the community controls was comparable to the

longitudinal samples taken from the febrile patients with an undetermined pathogen (Figure 5.2A), and those with culture confirmed *S. Typhi* and *S. Paratyphi A* on the day of admission ( $p>0.05$  for all comparisons, Wilcoxon signed-rank test) (Figure 5.2B&C). There was however a significant difference in anti-toxin IgG concentrations over the three-month sampling period in the enteric fever patients ( $p<0.05$ , Wilcoxon matched-pairs signed-rank test). Initially, the anti-toxin IgG responses in patients with *S. Typhi* and *S. Paratyphi A* were comparable, increasing between Day 1 and Day 8 and increasing again between Day 8 and Month 1 ( $p<0.05$  for both comparisons, Wilcoxon matched-pairs signed-rank test). However, anti-toxin IgG in plasma from the *S. Paratyphi A* patients declined after Month 1, plateauing to a Day 8 equivalent concentration at the three-month time point (Figure 5.2B).

The *S. Typhi* patients exhibited a differing anti-toxin IgG response, increasing from Day 8 to Month 1, and increasing again to the three-month time point ( $p<0.05$ , Wilcoxon matched-pairs signed-rank test) (Figure 5.2C). In total, 19% of the significant patterns of the longitudinal antibody profiles in the febrile patients with an undetermined pathogen exhibited sero-conversion against typhoid toxin, the equivalent proportion in those with *S. Typhi* or *S. Paratyphi A* was 80%.

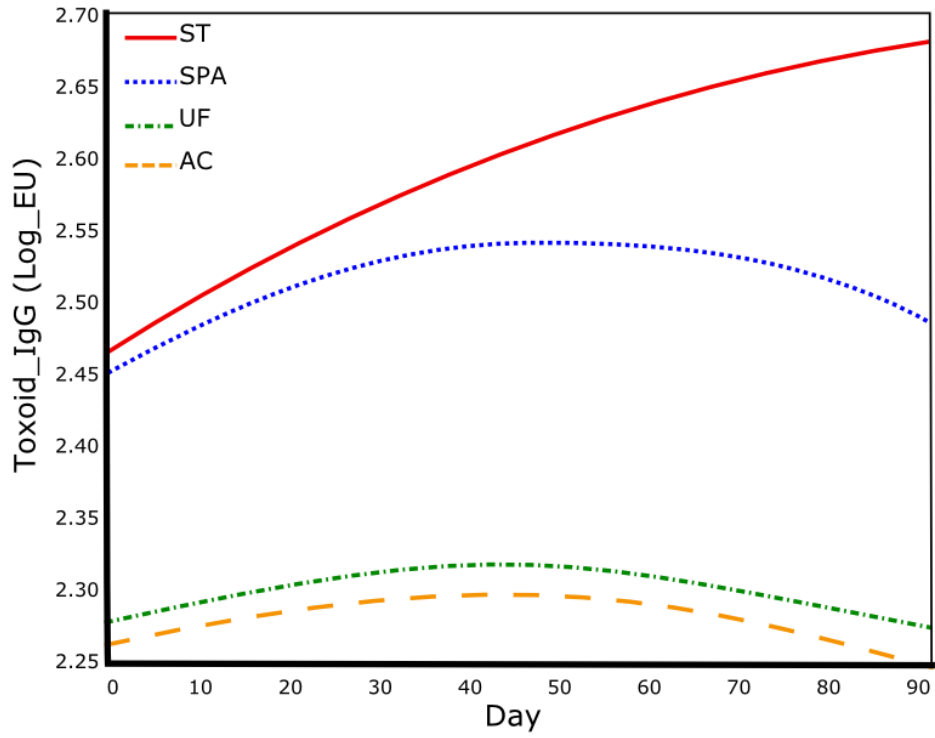


**Figure 5.2** Acute and convalescent IgG response to typhoid toxin

Boxplots showing the IgG antibody response against toxin (log<sub>10</sub> EU) up to three months after a febrile disease with A) an undetermined pathogen, B) *S. Paratyphi A*, and C) *S. Typhi*. Dark horizontal lines represent the mean, with the box representing the 25<sup>th</sup> and 75<sup>th</sup> percentiles, whiskers represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles; outliers are represented by dots. The dashed lines across the boxes represent the trend of the data throughout different time points and the shaded area around trend is 95% confidence interval. The solid/dashed lines above boxes define significantly different distributions ( $p \leq 0.05$ ) or not ( $p > 0.05$ ). AC: afebrile controls, D1: Day 1, D8: Day 8, M1: Month 1, M3: Month 3.



To better disaggregate the dynamics of anti-toxin IgG in the various patient groups, I fitted the longitudinal serological data using mixed effects model. The resulting patterns from the mixed effects model confirmed my observations of longitudinal anti-toxin IgG dynamics. The anti-toxin IgG trend line in the community controls and febrile patients with an undetermined pathogen were low, horizontal, and substantially divergent from those of the *S. Typhi* and *S. Paratyphi A* culture confirmed patients (Figure 5.3). However, anti-toxin IgG in the *S. Typhi* and *S. Paratyphi A* patients increased between Day 1 and Day 28; anti-toxin IgG subsequently decreased in the *S. Paratyphi A* patients and continued to increase in the *S. Typhi* patients at three-months post infection.



**Figure 5.3** The longitudinal dynamics of IgG response against typhoid toxin.

Mixed effects model using the anti-toxin IgG data from the afebrile control (AC, orange) for generating trends of anti-toxin IgG of those with an undetermined pathogen (UF, green), *S. Paratyphi A* (SPA, blue) and *S. Typhi* (ST, red) culture confirmed patients throughout 90 days of observation.

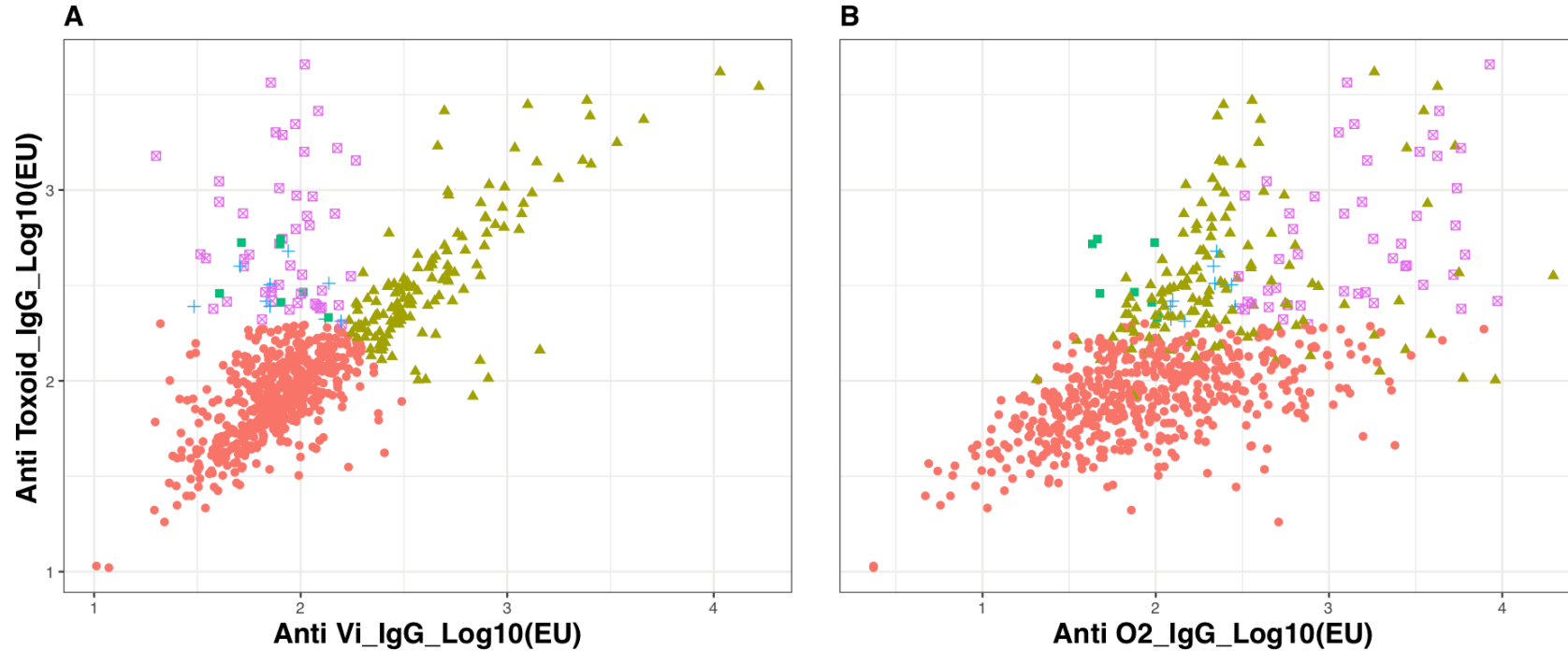
### 5.2.3 Anti-toxin and anti-Vi polysaccharide IgG responses in the general Nepali population

Aiming to examine the association between the humoral response to typhoid toxin and the Vi capsular antigen, I measured anti-toxin IgG and anti-Vi IgG antibodies in a cross-sectional plasma bank from afebrile Nepali individuals. Antibody titres against these two antigens were plotted and could be grouped into three distinct serological populations using K-means clustering (Figure 5.4A). The majority of individual plasma samples (669/733, 91.2%) exhibited a high correlation ( $\rho > 0.85$ ,  $p < 0.05$ , Spearman's correlation coefficient test) between anti-toxin IgG and anti-Vi IgG. The remaining data points (64/733, 8.8%) were skewed towards higher concentrations of anti-toxin IgG.

To assess potential exposures to *S. Typhi* in individuals in the plasma bank samples I considered the correlation between anti-toxin IgG and anti-Vi IgG titres within each of the three serological defined populations. The longest anti-toxin IgG responses for which I had data were in acute typhoid patients three-months post presentation, therefore any inference of *S. Typhi* exposure in the plasma bank was made on the assumption that it occurred within the three-month pre-sampling period.

In population one (532/733, 72.5%) (Figure 5.4A, red circles), anti-toxin IgG and anti-Vi IgG were proximal to the root of both axes, suggesting that individuals in this cluster did not have an antibody response indicative of recent *S. Typhi* exposure. In contrast, high concentrations of anti-toxin IgG and anti-Vi IgG in population two (137/733, 18.7%) (Figure 5.4A, green triangles) was indicative of *S.*

Typhi exposure within the proceeding three months. The profile in population three (64/733, 8.8%) (Figure 5.4A, pink checked boxes and green squares and blue crosses) contained those with lower anti-Vi IgG than those in population two and higher anti-toxin IgG than in population one.



**Figure 5.4** Correlations between antibody IgG responses to typhoid toxin and Vi and O<sub>2</sub> antigens.

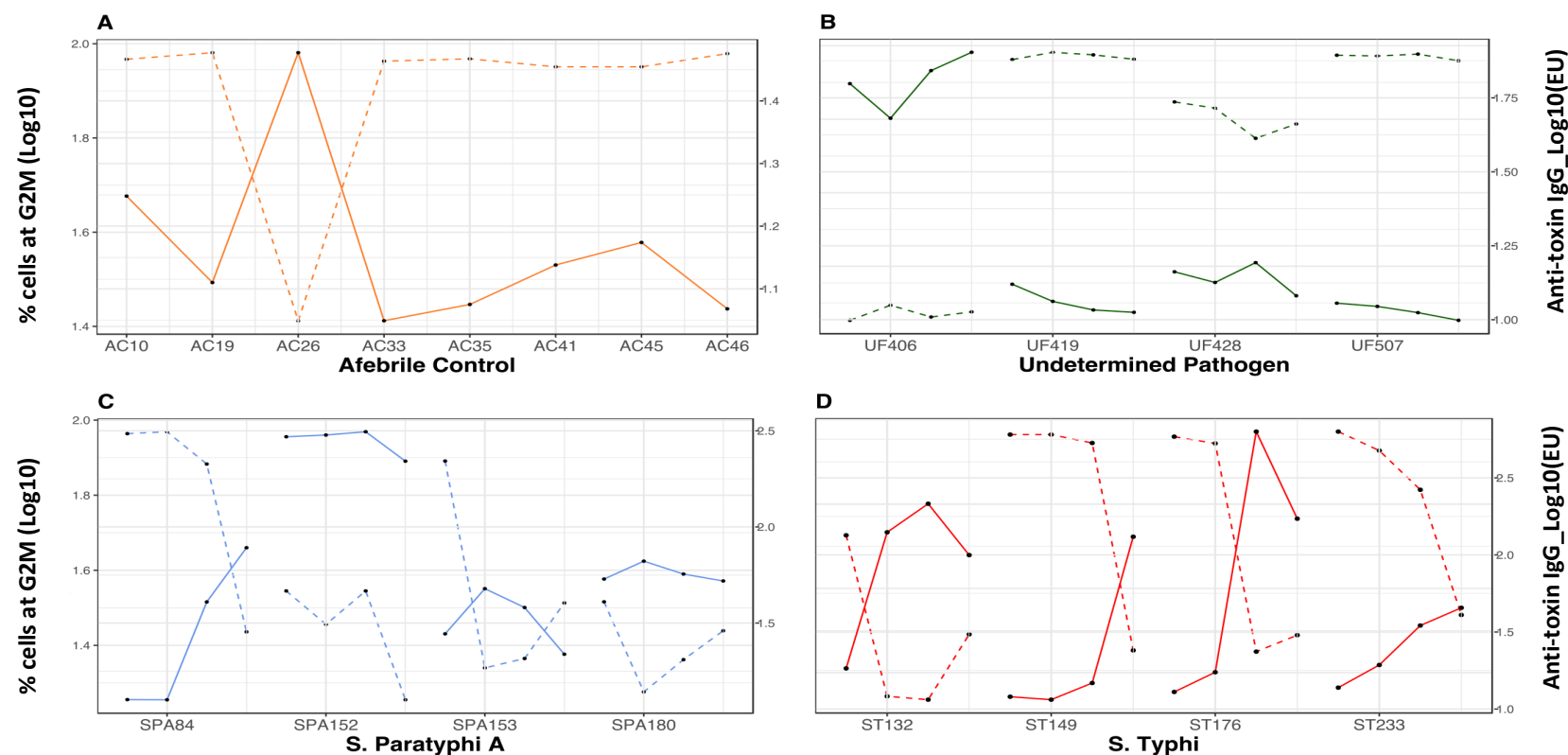
The associations between IgG response against typhoid toxin and Vi (A); or typhoid toxin and O<sub>2</sub> (B) in a Nepali plasma bank visualized by scatter plots. A) Every sample in the plasma bank was grouped into one of three population clusters by K-mean clustering method, these were population one of low anti-toxin IgG/low anti-Vi IgG (532/733, 72.5%, red circles), population two of high anti-toxin IgG/ high anti-Vi IgG (137/733, 18.7%, green triangles), and population three of high anti-toxin IgG/low anti-Vi IgG (64/733, 8.8%, pink checked boxes and green squares and blue crosses). B) Individuals in figure A are presented in relation with their values of anti-O<sub>2</sub> IgG, which reveals 75% (48/64, pink checked boxes) of population three having anti-O<sub>2</sub> IgG > 75<sup>th</sup> percentile, 14% (9/64, blue crosses) of population three having 50<sup>th</sup> percentiles <anti-O<sub>2</sub> IgG< 75<sup>th</sup> percentiles and 11% (7/64, green squares) of population three having anti-O<sub>2</sub> IgG < 50<sup>th</sup> percentiles.

I next hypothesized that the disparity between anti-toxin IgG and anti-Vi IgG in population three (high anti-toxin IgG/low anti-Vi IgG) was associated with *S. Paratyphi A* infection. To test the hypothesis, I additionally measured anti-O2 IgG in the plasma bank. I found that 48/64 (75%) of individuals who had high anti-toxin IgG/low anti-Vi IgG, had anti-O2 IgG concentrations in the 75<sup>th</sup> percentiles of the anti-O2 IgG concentrations in the plasma bank. Overall, the anti-O2 IgG titres exhibited a low degree of correlation ( $\rho \sim 0.5$ ,  $p < 0.05$ , Spearman's correlation coefficient test) against anti-toxin IgG titres (Figure 5.4B). The location of the 48 individuals with high anti-toxin IgG, low anti-Vi IgG, and high anti-O2 IgG (Figure 5.4B, pink checked boxes) were within the group with the highest levels of both anti-toxin IgG and anti-O2 IgG (Figure 5.4B). This observation suggested that *S. Paratyphi A* exposure may contribute to at least 75% of serological population 3, which was equivalent to 6.5% (48/733) of the entire serum bank and indicative of a lower incidence of infection than *S. Typhi*.

#### **5.2.4 Neutralization of typhoid toxin with acute and convalescent patient plasma**

My work demonstrated that a substantial longitudinal antibody response against typhoid toxin is mounted during and after infection with both *S. Typhi* and *S. Paratyphi A*. To assess if these anti-typhoid toxin antibody responses had any functional a toxin neutralization assay was performed. The neutralization assays were conducted on longitudinal plasma samples from four seroconverting participants (according to significant patterns) from each of the patient groups (*S. Typhi* confirmed, *S. Paratyphi A* confirmed, and febrile patients with an undetermined pathogen); eight afebrile community plasma samples were added as a

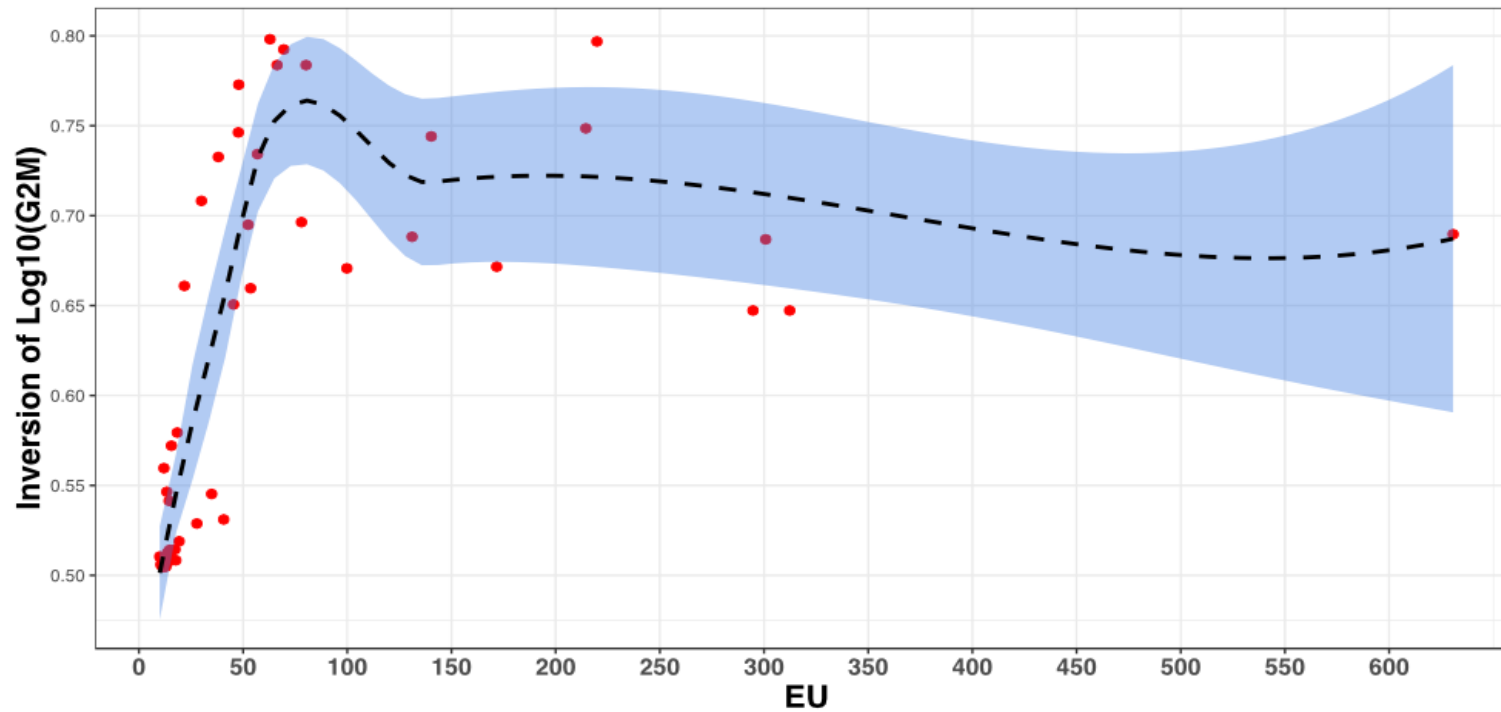
control. I could identify a robust toxin-neutralizing activity in all samples assayed that were obtained at Day 28 or longer after the onset of symptoms. In some patients, toxin neutralizing activity was detected as early as 8 days after infection (Figure 5.5A-D). A potential correlation between plasma neutralization capacity and anti-toxin IgG was visualized by plotting the inversion of cells arrested in phase G2M against anti toxin IgG concentration (Figure 5.6). We observed a good correlation ( $\rho \sim 0.85$ ,  $p < 0.05$ , Spearman's correlation coefficient test) between anti-toxin IgG and neutralization for plasma samples in which the anti-toxin IgG was  $< 100$  EU. However, there was no such correlation between anti toxin IgG and neutralization for plasma samples in which the anti-toxin IgG was  $\geq 100$  EU. This segregation in the capacity of human plasma to neutralize typhoid toxin suggests a saturation effect. The maximum neutralization ability of toxin-specific antibodies was reached at approximately 100 EU, whereby the amount of inoculated typhoid toxin was presumably all neutralized.



**Figure 5.5** The ability of human plasma from typhoid patients to neutralize typhoid toxin

Toxin neutralization was performed by examining the ability of different plasma samples to block the DNA damage response induced by typhoid toxin in cultured epithelial cells, which was figured by the number of arrested cells in the G2M phase of cell cycle in the intoxication experiment. Plots A-D represent the percentage of arrested cells at G2M phase of cell cycle. Right Y-axis was constructed by log scale of anti-toxin IgG in ELISA Unit of the plasma samples in community (A), and in undefined febrile (B), *S. Paratyphi A* (C) and *S. Typhi* (D) patients. Values of the right Y-axis are shown in solid lines. Left Y-axis was constructed by log scale of the percentage of arrested cells at G2M phase of cell cycle. Values of the left Y-axis are shown in dashed lines.





**Figure 5.6** Association between toxin neutralization ability and anti-toxin IgG concentration

Y-axis was constructed by Inversion of log scale of the percentage of arrested cells at G2M phase of cell cycle (IG2M). The Spearman's correlation ( $\rho$ ) between the level of antibodies and IG2M reached to 0.85 ( $\rho < 0.01$ ) when antibody titre was less than 100 EU and  $\rho$  approached to zero when the level of antibody exceeded 100 EU.

### 5.3 Discussion

Typhoid is still a life-threatening disease, especially in endemic parts of Africa and South Asia where the poverty compounds the modest circumstance of current typhoid diagnosis and treatment <sup>76</sup>. Better case detection is a principal aspect that should be considered for combating typhoid, followed by the need for an alternative/assistant therapy for typhoid treatment because of the sharp rise in reduced susceptibility and resistance to fluoroquinolone and other antimicrobials among *S. Typhi* and *S. Paratyphi A* strains globally <sup>43,46,50</sup>. The recent discovery of typhoid toxin and the various studies on its activities have revealed its potential for both diagnosis and treatment <sup>139,415</sup>. However, the experiments determining the function of typhoid toxin were performed either *in vitro* with human cell lines or in non-human primates (chimpanzee); therefore, we need further data and functional evaluation in human, the actual species where typhoid arises. The active subunit of typhoid toxin (cdtB) was recently assessed for the first time for its ability to classify typhoid patients and generated some promising results as an application for diagnosis <sup>333</sup>. Conducting additional investigations using longitudinal samples from typhoid patients (>200 enteric patients and >300 patients with undefined febrile disease) I investigated the acute and longitudinal immunological responses to typhoid toxin; the resulting data suggests that antibody against typhoid toxin is functional and may be suitable as a future approach for typhoid treatment.

Typhoid toxin is a virulence factor which is thought to be highly associated with early symptoms of typhoid <sup>138</sup>. I found that acute anti-toxin IgM responses were comparable between patients infected with *S. Paratyphi A* and *S. Typhi*. Notably, these antibody titres were significantly different and generally greater degree than

those for febrile patients with an unidentified pathogen. The possibility of having culture negative typhoid cases in the undiagnosed febrile patient group, which I used as the control for our classification, might have led to the reduction in specificity and sensitivity compared to those in the earlier study on acute immune response to CdtB<sup>333</sup>. I obtained better discrimination for *S. Typhi* infected cases when anti-CdtB IgM profile was combined with anti-Vi IgM<sup>333</sup>. This result may be a consequence of recompense for weakness of the induced IgM between polysaccharides and proteins<sup>333</sup>. Seemingly, I achieved a better typhoid case classification when combining acute antibody responses against typhoid toxin with Vi and O2.

Given that the typhoid toxin is shared between *S. Typhi* and *S. Paratyphi A* (and limited other *Salmonella* serovars) it appears that this may be a prime candidate for a diagnostic marker. The data suggests that the majority of patients have an early and prolonged IgG antibody response when infected with either *S. Typhi* and *S. Paratyphi A*. Consequently, these data are encouraging and suggest that any early IgG response may be suitable marker for the serological detection of enteric fever. The data presented here need to be replicated in additional cohorts and the utility of this approach should be tested in comparison to other diagnostic methods and the gold standard of blood culture.

The time duration for samples from patients was three months, which made this study a valuable insight into the longitudinal, long-term antibody responses to typhoid toxin. The trends of longitudinal response to typhoid toxin were marginally different between *S. Paratyphi A* and *S. Typhi* patients, providing better recognition for *S. Typhi* over *S. Paratyphi A*. The different distribution of the immunological responses against typhoid toxin between *S. Paratyphi A* and *S. Typhi* patients at

three months post onset of disease suggests longer term immunity to typhoid toxin in the later patient group. This observation also suggests that *S. Typhi* may have a distinctive ability for using the toxin as a key virulence factor, despite the toxin and its supportive proteins encoded by both *S. Typhi* and *S. Paratyphi A*. All of the experimental studies on typhoid toxin, including the data presented here, have been performed by using typhoid toxin purified from *S. Typhi*, which may have left specific signatures that make it better recognized by the immune system in those infected with *S. Typhi* than *S. Paratyphi A*.

An examination into the serum bank revealed that anti-toxin IgG correlated well with anti-Vi IgG (in the majority of the serum bank) but to lesser degree with anti-O2 IgG. Vi and O2 have been traditionally used to serologically detect *S. Typhi* and *S. Paratyphi A*, respectively <sup>264</sup>. The inclusion of anti-O2 IgG in addition to anti-toxin IgG and anti-Vi IgG on the serum bank samples provided evidence that anti-toxin IgG is preferable over Vi and O2 for detecting typhoid exposures. A potential downstream application of these data may be for estimating disease burden in locations with endemic typhoid for epidemiologic surveillance. This approach is ideal as it provides a specific tool that can detect exposure to either of the causative agents of enteric fever.

In this chapter I not only confirmed typhoid toxin as an immunogenic antigen of *S. Typhi* and *S. Paratyphi A* but also demonstrated the toxin-induced antibody is capable of neutralizing toxin. Because typhoid toxin has been demonstrated to be responsible for the death-dealing clinical symptoms of the disease <sup>138</sup>, my results are a significant step towards targeting typhoid toxin as a specific therapeutic through

monoclonal antibodies. Further, deactivated typhoid toxin could be considered as a component of enteric bivalent vaccines directing both *S. Typhi* and *S. Paratyphi A*.

My study may have generated a higher specificity and sensitivity to detect typhoid patients using anti-toxin IgM if classification was performed in a group of febrile patients with a confirmed diagnosis of other (non-*Salmonella*) pathogens. Therefore, using typhoid toxin for the purpose of developing a serological diagnostic test needs further investigation. While these data describe the antibody response dynamics to typhoid toxin up to three months after the onset of presentation, it would have delivered more insight for the enteric fever immunity if I had had access to additional samples collected at more prolonged time points.

In conclusion, I have assessed the acute and convalescent humoral immunity responses to typhoid toxin in a longitudinal plasma collection of Nepali people. My informative data offers supportive evidence for generating an antitoxin approach for enteric fever (caused by both *S. Typhi* and *S. Paratyphi A*) treatment. Furthermore, my work creates the foundation to support further to use typhoid toxin in diagnosis of enteric fever and in vaccine formulations against enteric fever.

## **Chapter 6 Insights into the immunogenicity of *Salmonella* Typhi protein antigens**

### **6.1 Introduction**

Increasing antimicrobial resistance in *S. Typhi* and *S. Paratyphi* A infections in endemic areas suggest that bivalent vaccine may be a more sustainable approach for enteric fever control <sup>416</sup>. Conjugate vaccines for enteric fever have been shown to be as safe and more efficacious than traditional unconjugated polysaccharide subunit vaccines <sup>135</sup>. The commonly used carrier proteins (e.g., rEPA, diphtheria toxoid, tetanus toxoid, and CRM<sub>197</sub>) convert polysaccharides (*S. Typhi* Vi or *S. Paratyphi* A O-specific polysaccharide) into T-cell inducers, but the polysaccharide antigens that would be used in these vaccines are heterologous between serovars. A homologous *S. Typhi*/*S. Paratyphi* A protein antigen that can be conjugated to *S. Typhi* Vi capsular and/or *S. Paratyphi* A O antigen polysaccharide may enhance a cross-reactive protective immune response. Consequently, this approach may induce greater efficacy than existing heterologous conjugate vaccines <sup>314</sup>, but this approach has not been progressed or tested in humans <sup>314</sup>. Therefore, a conjugate vaccine containing the Vi antigen or the *S. Paratyphi* A O antigen polysaccharide linked to a homologous protein antigen that can induce protection that is shared between the two serovars is an appealing concept to generate a bivalent vaccine against both forms of enteric fever. However, there are little data regarding the immunogenicity of *S. Typhi* and *S. Paratyphi* A protein antigens during natural infection.

Aiming to generate more insight into immunogenic repertoire of *S. Typhi* protein antigens, here I challenged a *S. Typhi* transposon mutant library with longitudinally plasma samples collected from enteric fever patients after the onset of infection to identify mutants that were able to survive serum bactericidal activity. I subsequently performed transposon directed-insertion site sequencing (TraDIS) on the surviving mutants to identify the specific proteins antigens that mediate antibody killing. To validate the resulting TraDIS data I sub-selected a smaller collection of TraDIS-identified *S. Typhi* antigens to assess their ability of inducing antibody mediated killing. Four of these antigens were able to induce bactericidal activity of both *S. Typhi* and *S. Paratyphi A*. I next aimed to describe the dynamics of the antibody response against these immunogenic antigens by measuring acute and convalescent antibody (IgG) against this selection of *S. Typhi* antigens in a cohort of typhoid and paratyphoid patients in Nepal. I identified a number of antigens that had sustained antibody response and that could induce bactericidal activity; thereby, suggesting their utility in a pan-enteric fever vaccine.

## **6.2 Results**

### **6.2.1 TnTMDH5 mutant library**

A mutant library consisting of approximately 500,000 mutants was generated using a Tn5-derived transposon using a clinical H58 *S. Typhi* isolate (AS252). The library was explored using short-read sequenced using transposon-specific primers (transposon directed-insertion site sequencing, TraDIS) to identify all the specific insertions. The recovered sequences determined that the TraDIS transposon library contained 202,982 unique insertion sites, resulting (on average) of a single insertion every 25bp of the genome (genome~4,800,000bp) (see input library in Table 6.1).

The insertion index, which equates to the number of unique insertion sites of any given gene divided by the gene length, had binomial distribution across the genome. This was comparable to a previously generated *S. Typhi* TraDIS library in the attenuated *S. Typhi* Ty2-derived strain CVD908-htrA<sup>343</sup>.



**Table 6.1** General information of TraDIS post typhoid plasma challenges

Pool of mutants/ Time of plasma collection	Total reads	Reads mapped to CT18 (%)	Unique insertion site	No of genes having Log2FC>2, $\rho$ value<10 <sup>-5</sup>
Input	2,312,856	93.8	202,982	
Output Day 1	2,535,040	93.54	8,219	292
Output Day 8	3,480,955	93.81	5,913	614
Output Month 1	2,793,832	93.82	37,197	81
Output Month 3	2,731,170	93.22	13,905	204

## **6.2.2 Bactericidal assay against the TnTMDH5 mutant library**

### **6.2.2.1 *Salmonella* Typhi antigens as a target for antibody mediated killing**

The generated transposon mutant library was subjected to challenge with plasma collected from enteric fever patients at different time points during clinical disease: Day 1, Day 8, Month 1, and Month 3 after presentation of illness (named Day 1, Day 8, Month 1, and Month 3 from this point forward). The library was challenged with plasma for 90 minutes and then plated on LB agar plates overnight, persisting bacteria were subjected to DNA extraction the following day. This procedure allowed the growth of the mutants that were resistant to the bactericidal activity of plasma from enteric fever patients, whilst eliminating or inhibiting susceptible mutants. This process was performed in parallel with equivalent experiments where the same pool of plasma at each time point was inoculated onto the wildtype parent *S. Typhi* to act as a control. This control was performed to ensure that the experimental dilution of pooled plasma was capable of killing 100% of the wildtype organisms. Therefore, any mutants that were resistant to bactericidal challenge were likely to be generated as a result of them lacking the specific target antigen.

The plasma samples were collected from patients participated in a clinical trial comparing two fluoroquinolone antimicrobials for the treatment of enteric fever. Therefore, there may be a concern of residual antimicrobials in the plasma samples which may affect the results of the bactericidal assays. However, the resulting data (presented below) suggest these antimicrobials did not interfere with the bactericidal activity because mutations were not identified in the site these antimicrobials are active.

The recovery rates (the proportion of organisms at each time point) of the transposon mutant library post plasma challenges are shown in Table 6.2 and the numbers of recovered sequences are shown in Table 6.1. The sequences of the plasma-challenged output transposon libraries were compared to those of the input transposon library at each time point to identify the loci encoding antigens required for antibody mediated killing. Essentially, the interruption of genes with a transposon insertion generated a set of clones that could survive the challenge with plasma from typhoid patients. This process enriched specific genes in the output pool in comparison to the input pool. Genes that were significantly increased in the output pool ( $\text{Log}_2\text{Fold-Change} \geq 2$  and  $p$  value  $< 10^{-5}$ ) were considered to be relevant for further investigation.

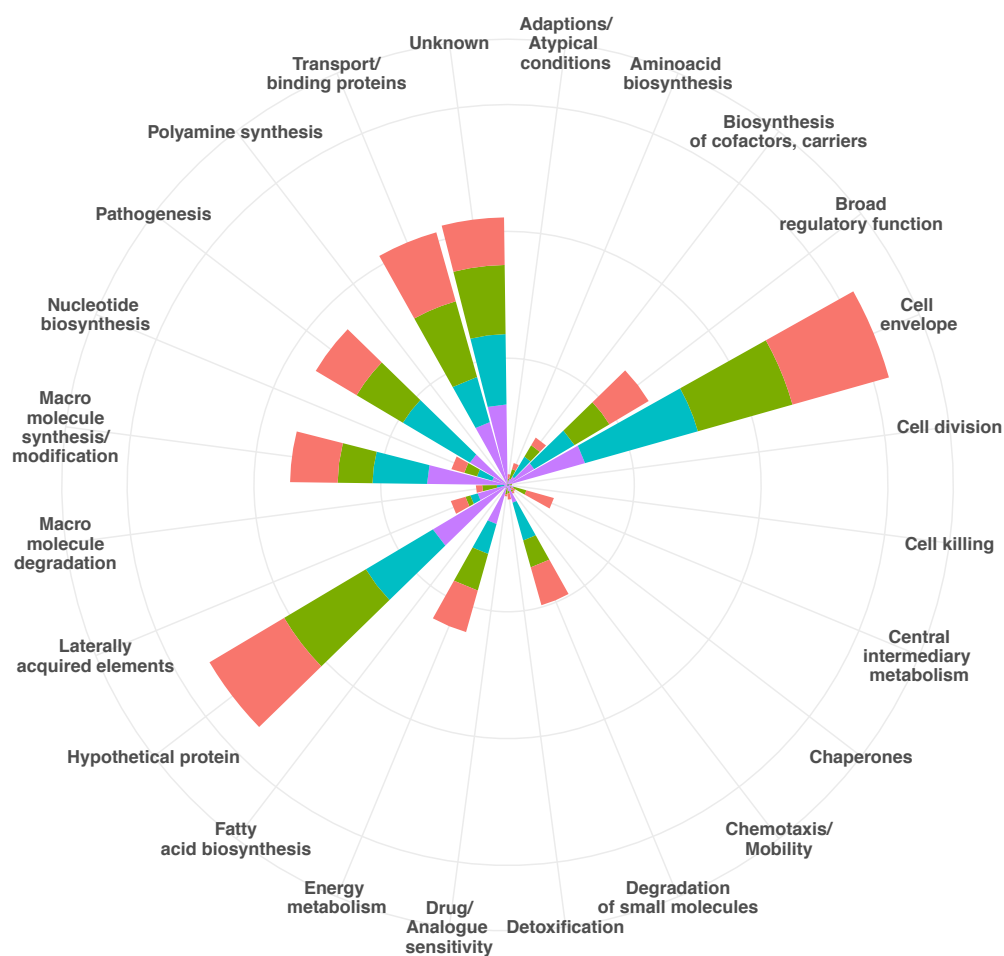
**Table 6.2** The bacterial recovery rate of TraDIS post challenge with typhoid plasma

Time of plasma collecting	% Bacterial recovery compared to control	
	Wild-Type <i>S. Typhi</i>	Transposon mutant <i>S. Typhi</i>
Day 1	0%	0.05%
Day 8	0%	0.01%
Month 1	0%	0.28%
Month 3	0%	0.06%

#### **6.2.2.1.1 Molecular functional classes and KEGG pathways**

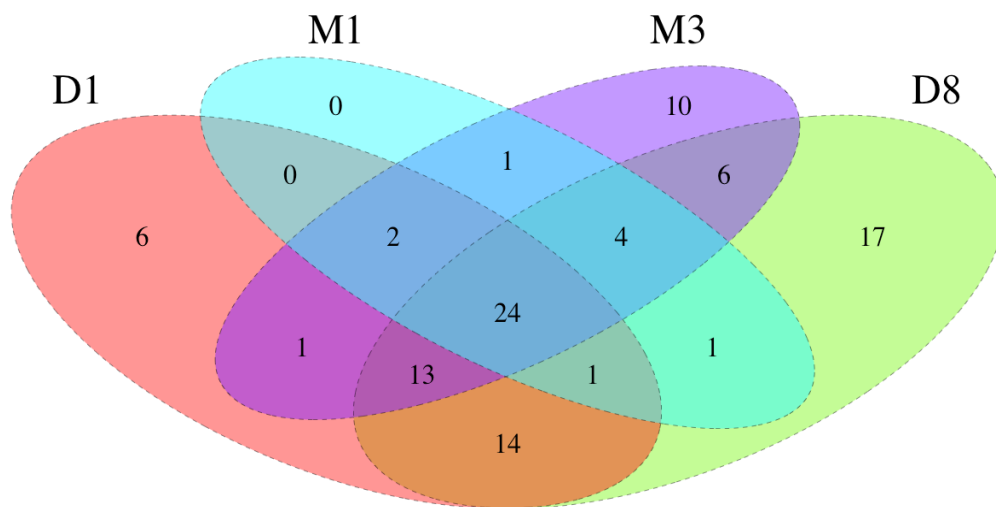
After challenging the transposon mutant library with Day 1, Day 8, Month 1 and Month 3 typhoid patient plasma, I identified 292, 614, 81, and 204 candidate mutants that respectively survived the challenge at the specified significance level ( $L2FC \geq 2$  and  $p$  value  $< 10^{-5}$ ) (Appendix A). The candidate gene collections identified at Day 1, Day 8, Month 1 and Month 3 were classified according to previously assigned functional classes (Table 2.7).

The most common and shared molecular functional classes identified in the output pools at the various time points were genes involved in cell envelope, transport/binding proteins, pathogenicity, broad regulatory proteins, and genes encoding a large quantity of unknown and hypothetical proteins (Figure 6.1). These candidate genes were also categorised into various biological pathways using the KEGG pathways system<sup>417</sup> (Figure 6.2). The majority of identified pathways were shared between two, three, or all four of the time points. The most prominent biological pathways identified were involved in infection (sty05132), quorum sensing (sty02024), bacterial chemotaxis (sty02030), flagella assembly (sty02040), peptidoglycan biosynthesis (sty00550), lipopolysaccharide biosynthesis (sty00540), bacterial secretion (sty03070), purine metabolism (sty00230), and two-component regulation (sty02020). Additionally, the gene pool at each time point also identified specific biological pathways that may reflect the different phases of bacterial infection. Of note, the specific biological pathways identified on Day 8 and Month 3 were siderophore biosynthesis (sty01053), and fatty acid metabolism/ biosynthesis/ degradation (sty01212/ sty00061/ sty00071), respectively.



**Figure 6.1** Functional classification of *Salmonella* Typhi genes identified following typhoid plasma challenge of the *Salmonella* Typhi transposon mutant library

Functional classes are indicated on the outer most circle. Four circles from the middle represent 20 – 40 – 60 – 70 per cent of the cumulative percentage of functional classes over four time points. Purple, blue, green and red blocks are representatives of Month 3, Month 1, Day 8, Day 1 respectively.



**Figure 6.2** Shared KEGG pathways of *Salmonella* Typhi genes identified following typhoid plasma challenge of the *Salmonella* Typhi transposon mutant library

KEGG pathways fitted for identified genes from Day 1 (red oval), Day 8 (green oval), Month 1 (blue oval) and Month 3 (purple oval). The number of specific or shared pathways among time points are indicated by a number in a corresponding division.

#### 6.2.2.1.2 Candidate genes

Approximately 20 – 25% of all identified genes at each time point encoded either hypothetical or unknown proteins, opening great potential for further investigation. However, several groups of genes, which have been previously studied, were identified at specific time points or at all time points. Flagellar associated genes<sup>117</sup> such as *fliHKNMT*, *flgG*, and the torque-generating machinery, *motA*, and genes associated with invasion and the SPI-1&2 type III secretion systems, such as *invA*, *invF*, *pagC*, *prgh*, *spaM*, *sigD*, *sipB*, *sseB*, *sspH2*, *stpA* were also identified<sup>418–423</sup>. Notably, these key loci (and others as described in Appendix A) were detected at all four time points. Genes required for bacterial adherence to the host cells as well as those involved in bacterial motility, such as fimbriae (17 genes in total, including *fimF*, *fimW*, fimbrial subunits *bcfA*, *steF*, fimbrial chaperons *steC*, *stbB*, and *stgB*,<sup>123,424</sup>) and type IV pilus (4 genes, *pilABCT*,<sup>425,426</sup>) (Appendix A), were enriched at Day 1 and Day 8. Genes associated with the synthesis and export of siderophores (STY0631, STY0637, STY0639, STY0641)<sup>427–429</sup> and genes involving in Vi capsular polysaccharide synthesis, *tviABCDE* and *vexABE*<sup>118</sup>, were detected at Day 8 but not the remaining time points (Appendix A).

The gene pools of the mutants recovered from the typhoid plasma challenges revealed some overlap with the gene pools previously detected using a protein microarray that detected immunogenic antigens<sup>336</sup>. This overlap encompassed 46 genes over the four time points: Day 1, 9 genes; Day 8, 23 genes; Month 1, 3 genes; and Month 3, 11 genes (Table 6.3). Notably, 6 genes out of this overlap were also identified as having diagnostic potential in Chapter 4<sup>333</sup>; these genes were



STY3208, STY1372 (Day 1), STY1767\_*nlpC*, STY4190\_*yhjJ* (Day 8) and  
STY1612, STY1498\_*hylE* (Month 3).

**Table 6.3** Genes identified by protein array\* and by TraDIS following typhoid plasma challenges of the *Salmonella* Typhi transposon mutant library

GeneID_CT18	GeneID_ATCC9150	Gene name	Description	Antibody class**	Time
STY1372	SPA1195	pspB	phage shock protein B	IgM	D1
STY3008	SPA2743	sipB	pathogenicity island 1 effector protein	IgG	D1
STY2595	SPA0500	dedD	DedD protein	IgG	D1
STY0332	SPA2464	safA	probable lipoprotein	IgG	D1
STY2259	SPA0817	pduT	putative propanediol utilization protein PduT	IgG	D1
STY1871	NA	-	putative heat shock protein	IgG/IgM	D1
STY3208	SPA2920	-	hypothetical protein	IgG	D1
STY3684	NA	-	phage lysis regulatory protein, LysB family	IgG/IgM	D1
STY1767	SPA1500	nlpc	putative lipoprotein	IgG	D8
STY4190	SPA3469	yhjJ	putative zinc-protease precursor	IgG	D8
STY4519	SPA4136	phoN	nonspecific acid phosphatase precursor	IgG	D8
STY3970	SPA3658	ibpB/hslS	heat shock protein B	IgG	D8
STY2467	NA	sspH2	secreted effector protein	IgG	D8
STY2894	SPA2633	iroN	TonB-dependent outer membrane siderophore receptor	IgG	D8
STY4023	SPA3613	mgtB	Magnesium transport ATPase, P-type 2	IgG	D8
STY0231	SPA0215	htrA	protease DO precursor; heat shock protein HtrA	IgG	D8
STY1384	SPA1205	mppA	periplasmic murein peptide-binding protein MppA	IgG	D8
STY2179	SPA0899	fliH	flagellar assembly protein FliH	IgG	D8
STY3958	SPA3670	yhjA	probable cytochrome c peroxidase	IgG	D8
STY3969	SPA3659	hslT	heat shock protein A	IgG	D8
STY4260	SPA0206	ggt	putative minor fimbrial subunit; putative adhesin	IgG	D8
STY4721	SPA4181	hflC	HflC protein	IgG	D8

GeneID_CT18	GeneID_ATCC9150	Gene name	Description	Antibody class**	Time
STY3765	SPA3947	-	hypothetical protein	IgG	D8
STY4570	NA	traB	TraB pilus assembly family protein	IgG/IgM	D8
STY3837	SPA3881	-	putative lipoprotein	IgG	D8
STY0294	SPA2496	-	ClpB-like protein	IgG	D8
STY1368	SPA1191	sapB	peptide transport system permease protein SapB	IgG/IgM	M1
STY1498	SPA1306	hlyE	haemolysin HlyE	IgG	M3
STY0793	SPA1996	tolA	tolA protein	IgG	M3
STY0107	SPA0093	surA	survival protein SurA precursor	IgG	M3
STY3252	SPA2962	pilT	Type II secretion, ATP-binding, protein	IgG	M3
STY3178	SPA2898	-	conserved hypothetical protein	IgG/IgM	M3
STY1444	SPA1249	-	putative glycolate oxidase	IgG	M3
STY1612	NA	-	putative membrane protein	IgG/IgM	M3
STY0303	SPA2484	-	probable lipoprotein	IgG	M3
STY4745	SPA4206	yjfY	conserved hypothetical protein	IgM	D1
STY0065	SPA0057	oadG	oxaloacetate decarboxylase gamma chain	IgM	D8
STY3969	SPA3659	hslT	heat shock protein A	IgM	D8
STY4260	SPA0206	ggt	putative minor fimbrial subunit; putative adhesin	IgM	D8
STY3093	SPA2820	-	hypothetical protein	IgM	D8
STY3671	NA	-	possible lipoprotein	IgM	D8
STY2368	SPA***	-	Putative membrane protein	IgM	M1
STY0788	SPA2001	ybgT	putative membrane protein	IgM	M1
STY0682	SPA2102	tatE	sec-independent protein translocase protein TatE	IgM	M3
STY1969	SPA1034	-	putative exported protein	IgM	M3
STY4599	SPA2554	-	hypothetical protein	IgM	M3

\*Protein array <sup>336</sup>

\*\*Antibody classes immune-recognised by the protein array

\*\*\*STY2368 is a conserved protein among *Salmonella enterica*. It is a small protein (38 aa) on *Salmonella* Paratyphi A ATCC\_9150 genome, 100% matched to STY2368 but is not annotated.

NA. not available on *S. Paratyphi A* genome

D1: Day 1; D8: Day 8; M1: Month 1; M3: Month 3

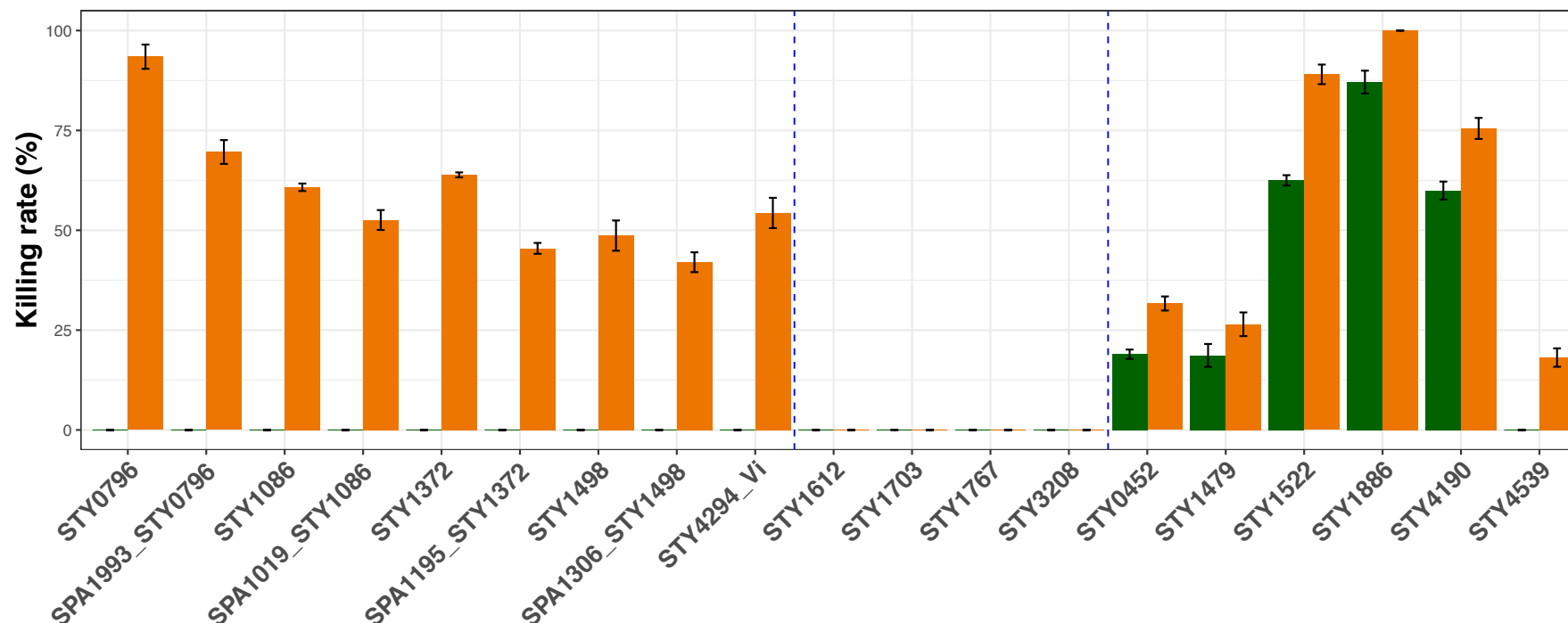
### **6.2.3 Antibody-dependent complement-mediated killing by mice plasma immunised with *Salmonella* Typhi antigens**

#### **6.2.3.1 Plasma directed *Salmonella* Typhi killing**

I aimed to validate the TraDIS data by investigating a selection of six candidate genes (STY3208, STY1372, STY1767\_*nlpC*, STY4190\_*yhjJ*, STY1612, and STY1498\_*hlyE*) that were identified by protein microarray and TraDIS. I aimed to assess if IgG against the selected antigens was functional for inducing specific bactericidal activity against *S. Typhi*. I hypothesized that plasma from animals immunised with specific antigens is capable of reducing or restricting the growth of a wild type *S. Typhi*. Further, I hypothesised that mutants lacking the specific gene encoding the target antigen (dMT) would not be subjected to the same bactericidal activity. I additionally extended this experiment to the other antigens that I evaluated for diagnostics (STY0452, STY0796, STY1086, STY1522, STY1703, STY1886, STY4539) and to other *S. Typhi* antigens known to be antigenic (STY1479\_*yncE*, Vi).

To test the above hypothesis, C57BL mice were independently immunized with these 14 protein antigens, the Vi polysaccharide antigens and PBS. Plasma from each of the mice immunized with the specific antigens were used to challenge the WT *S. Typhi* and the corresponding isogenic dMT mutant. Plasma from mice immunised with PBS was used as a control for bactericidal assay. Plasma from mice immunised with PBS demonstrated some intrinsic bactericidal activity against the wild type strain and each of the mutants, this killing activity was comparable between the wild type and mutants and ranged from 0% to 4% (data not shown). This correction factor was used for each antigen to control for the effect of intrinsic

bactericidal activity. The results of the bactericidal assays with plasma from all 15 antigen immunisations could be categorized into three groups (Figure 6.3). The first group (n=5) contained the plasma from the mice immunised with STY0796, STY1086, STY1372, STY1498 and Vi. All could induce bactericidal killing with WT *S. Typhi* but not (or low level) to the paired dMT (i.e. an isogenic organism lacking the gene encoding the target protein). The second group (n=4) contained the plasma from the mice immunised with STY1612, STY1703, STY1767, and STY3208 failed to induce bactericidal activity to both the WT and the paired dMT. Finally, the third group (n=6) contained plasma from the mice immunised with STY0452, STY1479, STY1522, STY1886, STY4190 and STY4539. These plasma samples showed a comparable degree of bactericidal activity between WT and dMT or exhibited no bactericidal activity against the dMT but low level killing against the WT.



**Figure 6.3** Bactericidal activity of immunised mice plasma on *Salmonella* Typhi and *Salmonella* Paratyphi A

Bacteria was challenged by mice plasma immunised with selected antigen-immunised individually. Killing rates, for a wild type parent strain (WT) in orange and for a descendant mutant strain (DMT) in green, were calculated as  $(1 - \text{ratio}(\text{number of SBA}_{\text{active\_complement}} \text{ recovered colonies} / \text{SBA}_{\text{inactive\_complement}} \text{ recovered colonies})) \times 100\%$ . The first group shows good bactericidal activity of *S. Typhi* (STY) and *S. Paratyphi A* (SPA) challenged by mice plasma immunised with four protein antigens (STY0796, STY1086, STY1372, STY1498) and Vi polysaccharide. The second group does not show any bactericidal killing to either WT or dMT *S. Typhi* challenged by mice plasma immunised with four antigens (STY1612, STY1703, STY1767, STY3208). The third group includes results of *S. Typhi* challenged by mice plasma immunised with six antigens (STY0452, STY1479, STY1522, STY1886, STY4190 and STY4539), which showed some degree of bactericidal activity to both WT and dMT.

#### **6.2.3.2 *Salmonella* Paratyphi A killing**

The aim of this series of experiment was to identify shared *S. Typhi* and *S. Paratyphi* A protein antigens that are immunogenic and able to be targeted by bactericidal activity. Consequently, I performed a comparable series of bactericidal assays challenging *S. Paratyphi* A as opposed to *S. Typhi*. I selected the plasma immunised with four antigens (STY0796, STY1086, STY1372, and STY1498) belonging to group one (Figure 6.3) as these plasma samples induced bactericidal activity against WT *S. Typhi*, but this was diminished or removed with the isogenic mutants. These plasma samples were used in a bactericidal assay against a wild type *S. Paratyphi* A and a corresponding mutant for each of the antigens. The results were consistent between *S. Paratyphi* A and *S. Typhi* (Figure 6.3). Briefly, plasma from mice immunized with STY0796, STY1086, STY1372 and STY1498 could induce killing against both WT *S. Paratyphi* A and *S. Typhi*, but this was diminished or completely removed against a mutant lacking the target antigen.

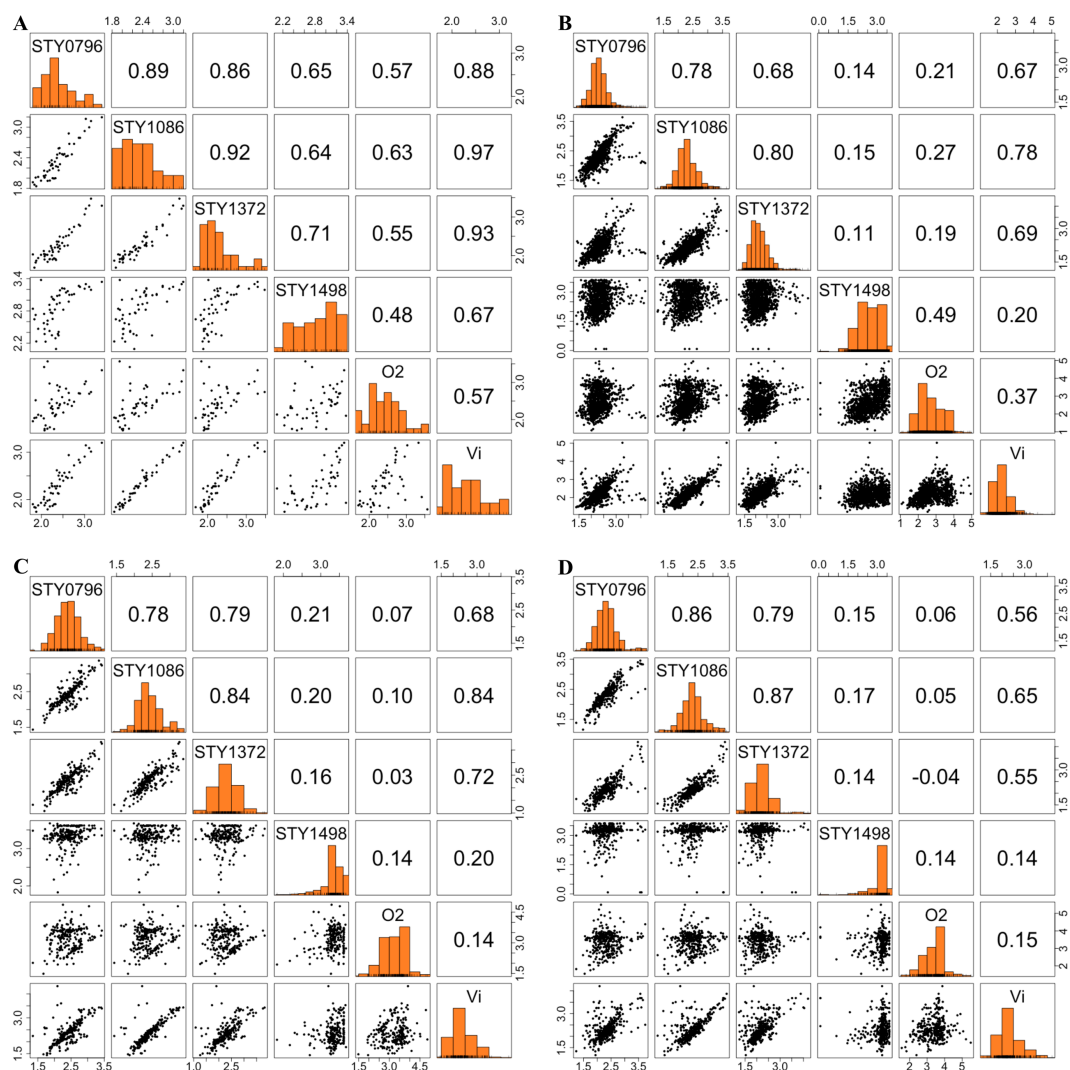
#### **6.2.4 *Salmonella* Typhi specific antibody dynamics in enteric fever patients**

Aiming to extend the data generated from the SBA experiment, I investigated the dynamics of specific antibody responses to the four antigens (STY0796, STY1086, STY1372, and STY1498), that could induce killing to *S. Typhi* and *S. Paratyphi* A, during natural enteric fever infections. I compared these antibody responses to those induced by STY1886, Vi and O2. ELISAs were independently performed to detect IgG antibodies against these 7 *S. Typhi*/*S. Paratyphi* A antigens on a collection of longitudinal plasma samples (up to three-months post presentation/admission) from enteric fever patients with culture confirmed *S. Typhi* and *S. Paratyphi* A infections (*S. Typhi*, n=152 and *S. Paratyphi* A, n=68). The same assays were performed on

plasma from febrile patients with an undetermined pathogen (n=316), and community controls (n=49).

The data generated from this series of ELISAs revealed that antibody could be detected longitudinal against all the selected antigens. Therefore, I firstly examined if there was a correlation between anti-antigen antibodies in plasma samples from different patient groups and the controls (Figure 6.4). Except for the anti-STY1498 antibody, the antibody responses against the remaining anti-antigen antibodies were well correlated to one another in all groups (control and patients); the value of rho was  $\geq 0.7$  ( $p < 0.05$ , Spearman's correlation coefficient test). Conversely, the antibody responses against the two polysaccharide antigens (Vi, O2) correlated poorly with the protein antigens and to one another.

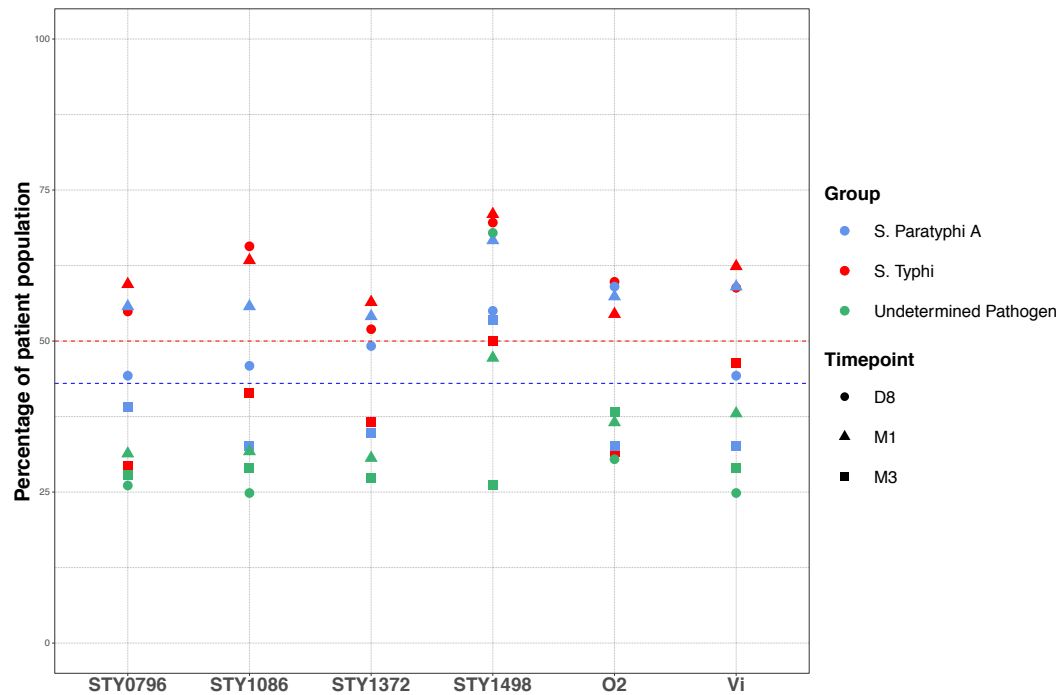




**Figure 6.4** Correlation between antibody responses to various *Salmonella* antigens

Spearman correlation among antigen-induced antibodies in (A) febrile controls unidentified pathogen febrile patients (B), *S. Paratyphi A* confirmed patients (C) and *S. Typhi* confirmed patients (D). The names of the various antigens and histograms showing the distribution of the antibody IgG against such antigens are presented on the diagonal. Scatterplots below the diagonal represent the correlation of IgG measurements of the two antigens on a right angle to the plots. The numerals above the diagonal depict the Spearman correlation coefficient ( $\rho$ ) values of the mirrored plots. OD values of each antigen-induced antibody are presented on side of the corresponding scatterplots.

Notably, more than 50% and 40% of patient population infected with *S. Typhi* or *S. Paratyphi A*, respectively, had detectable IgG against all antigens on Day 8 and Month 1. These responses were elevated by at least 15% in comparison to those measured on Day 1 (Figure 6.5). Conversely, those with afebrile controls and undetermined pathogen febrile patients did not elaborate a comparative antibody response.

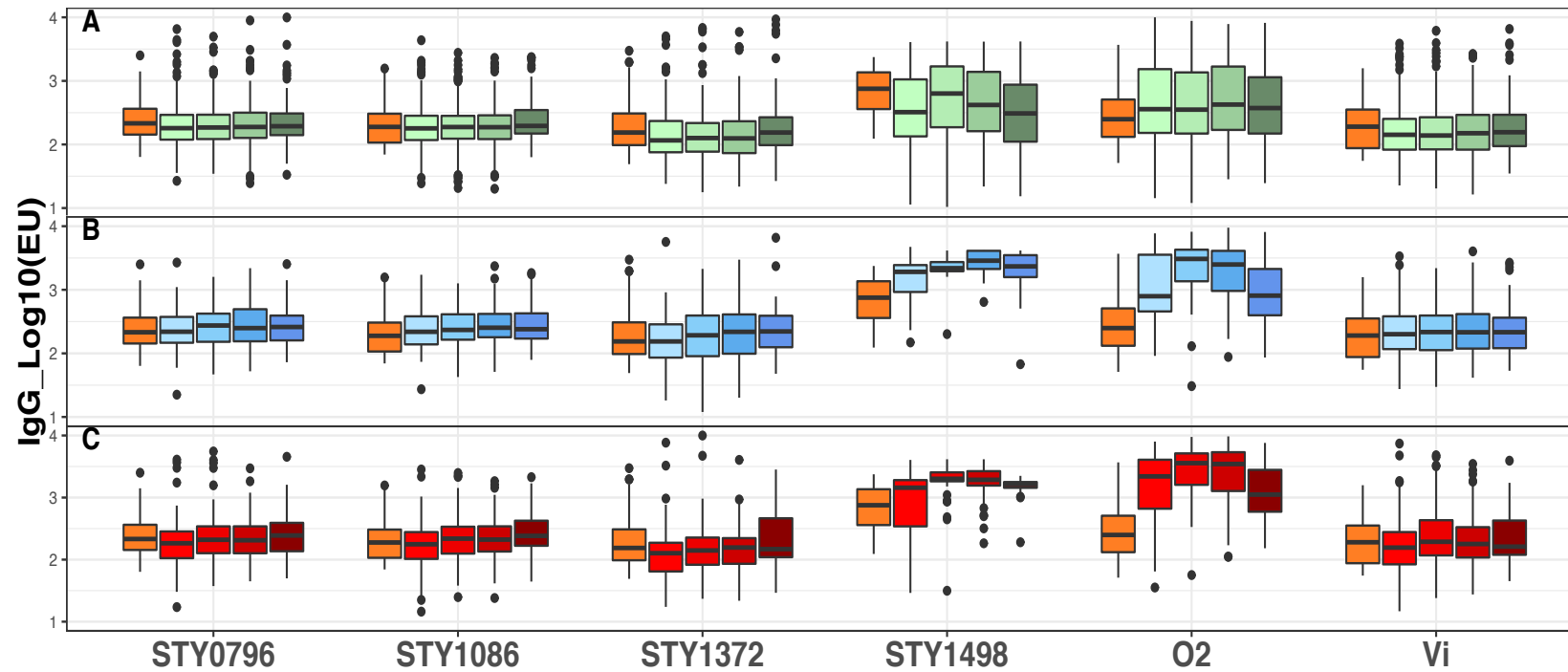


**Figure 6.5** Anti-antigen IgG measurements at threshold of 15% elevation in comparison to those measured on Day 1

Vertical axis represents the percentage of patient population having at least 15% elevation of IgG measurements in comparison to those measured on Day 1. Different shapes represent different time points; circles for Day 8, triangles for Month 1 and squares for Month 3. Colours indicate groups of patients; green for unidentified febrile patients, blue for *S. Paratyphi A* infected patients and red for *S. Typhi* infected patients. Blue and red dashed lines were constructed at 43% and 50%, respectively, of the vertical axis measurement unit.

On the day of admission, the concentrations of anti-antigen IgG in plasma from the three patient groups were found to be at a comparable level in plasma samples from afebrile community controls ( $p>0.05$ , Wilcoxon signed-rank test), this was true for all antigens (Figure 6.6). However, the general dynamic patterns of longitudinal anti-antigen IgG in plasma samples of three patient groups was different. Firstly, in the group of febrile patients with an unidentified pathogen (Figure 6.6A), the IgG response induced by the majority of the antigens (except STY1498) were longitudinally comparable to the baseline measurements recorded on Day 1 ( $p>0.01$ , Wilcoxon matched-pairs signed-rank test). Secondly, in the *S. Paratyphi A* confirmed patients (Figure 6.6B), in comparison to those at Day 1, the induced antibody IgG significantly increased at Day 8 (STY1498 and O2,  $p<0.05$ , Wilcoxon matched-pairs signed-rank test) or gradually increased achieving a statistically significant difference at Month 1 (STY0796, STY1086, STY1372,  $p<0.05$ , Wilcoxon matched-pairs signed-rank test); both trends were consistent until Month 3. Alternatively, IgG antibody against Vi was not significantly elevated over the reference/baseline measurements at any time point of sampling ( $p>0.05$ , Wilcoxon matched-pairs signed-rank test). Thirdly, in the *S. Typhi* confirmed patients (Figure 6.6C), the anti-antigen IgG concentrations in plasma samples (for all antigens, individually) were significantly elevated at Day 8, in comparison to those at Day 1, ( $p<0.05$ , Wilcoxon matched-pairs signed-rank test) and remained consistently high until Month 1. This was true of all antigens with the exception of IgG against O2 polysaccharide which declined substantially after Month 1 ( $p<0.05$ , Wilcoxon matched-pairs signed-rank test), plateauing to the concentration of Day 1 at Month 3 ( $p>0.05$ , Wilcoxon matched-pairs signed-rank test) (Figure 7.6C). Notably, antibody

against the other antigens was maintained at a stable and comparable level as those measured on day 8 until the end of the observation (STY0796, STY1498 and Vi,  $p < 0.05$ , Wilcoxon matched-pairs signed-rank test). Some IgG titres even rose again after Month 1 and significant differences between Day 8 at Month 3 titres were observed for STY1086 and STY1372 ( $p < 0.05$ , Wilcoxon matched-pairs signed-rank test) (Figure 6.6C).



**Figure 6.6** Longitudinal IgG responses against *Salmonella* Typhi/*Salmonella* Paratyphi A antigens in a Nepali cohort of febrile patients and controls

Three panels of boxplots showing IgG measurements (log10 of ELISA Unit) in plasma from febrile patients with an unidentified pathogen (A panel in green), *S. Paratyphi* A infected patients (B panel in blue) and confirmed *S. Typhi* infected patients (C panel in red). Every group of five boxplots presents IgG responses against a specific antigen, with the first box is the IgG measurement in the afebrile controls (orange), the 2<sup>nd</sup> to 5<sup>th</sup> boxes are the IgG measurement in the patient groups on Day 1, Day 8, Month 1 and Month 3, respectively. Antibody responses against STY0796, STY1086, STY1372, STY1498, O2 and Vi are displayed from left to right. Dark horizontal lines represent the mean IgG measurement, with the box representing the 25<sup>th</sup> and 75<sup>th</sup> percentiles, whiskers represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles; outliers are represented by dots.

### 6.3 Discussion

The current licensed vaccines against *S. Typhi* are likely to play an important role in the decline of typhoid worldwide <sup>430</sup>; however, they do not confer protection against *S. Paratyphi A* <sup>323</sup>. Furthermore, paratyphoid vaccine development is lagging behind the increase of reported paratyphoid cases <sup>323</sup>. Focussing on development of a monovalent vaccine for either of the typhoidal *Salmonella* serovars does not convey the real need for enteric fever control campaigns. Bi-valent vaccines against both *S. Typhi* and *S. Paratyphi A* would be a valuable public health tool to address this life-threatening disease more broadly <sup>416</sup>. The novel strategy of using a homologous protein carrier between serovars for the bi-valent conjugate vaccines should become a priority <sup>431</sup>, which may enhance the immune response against the targeted pathogens leading to extra effectiveness over conjugates using a heterologous carrier <sup>314</sup>.

The stimulation and prolongation of a protective antibody response of an immunogenic candidate antigen is crucial for the development of a successful vaccine. However, a more informed understanding of the host immune responses against *S. Typhi*/ *S. Paratyphi A* has proved challenging as these two typhoidal serovars are restricted human pathogens. Therefore, the antigens provoking a potentially host protective immune responses have been largely uninvestigated. The immunogenicity of a small number of *S. Typhimurium* and *S. Typhi* antigens, such as Fim C<sub>S</sub> <sup>432</sup>, outer membrane adhesion protein T2544 <sup>433,434</sup>, and OmpS1&2 <sup>435</sup>, have been studied in mouse models and extrapolated to typhoid infection in humans. However, the antigens that found to be immunogenic in murine models may not adequately recapitulate in *S. Typhi* natural infection <sup>436</sup>. Some studies have

investigated *in vitro* stimulation to human immune cells that were collected from post Ty21a immunized vaccinees by *S. Typhi* protein antigens such as SifA, OmpC, FliC, GroEL <sup>437</sup>. Other studies investigating the immunogenicity of *S. Typhi* antigens for vaccine potential based on the antigen-antibody recognition within natural human *S. Typhi* infected sera have also been implemented <sup>336,438,413</sup>. Yet, these studies stopped after confirming the presence of these antigen-antibody complexes in the typhoid patient plasma (differed from in healthy control plasma) without any investigation of their function. As *S. Typhi* and *S. Paratyphi A* are facultative intracellular bacteria, humoral and cell-mediated immunities harmoniously orchestrate the duty of eradicating extracellular bacteria and bacteria-infected cells <sup>439</sup>. Antibody-dependent complement-mediated killing (bactericidal activity) is one of two operative attributes (antibody-dependent phagocyte-mediated killing or opsonophagocytic activity is the other) of serum antibodies that have been recorded for estimation of protection potential of bacterial vaccines including for typhoid <sup>431,440–442</sup>.

My study performed a series of experiments to generate extensive information on the induction and function of antigen specific antibody (direct *S. Typhi*/ *S. Paratyphi A* killing) over time, after the original point of infection. The primary screening for immunogenic *S. Typhi* antigens were longitudinal plasma bactericidal activity, which delivered a unique data that provides fundamental data for future research on novel immunogenic antigens that may contribute to vaccine development. The most common and shared (between time points) molecular functions of the target antibody response were cell envelope, pathogenesis, quorum sensing, bacterial chemotaxis, and previously characterised genes involved in flagella biosynthesis.



These targets were identified throughout the course of investigation; confirming that functional and long-lasting antibodies are induced by antigens involved in the bacteria communicating with each other, the surrounding environment, and with the host. Additionally, fimbriae and pili are amongst the earliest virulence factors that bacteria exploit during infection, these were also found to induce specific and functional antibodies at early time points.

Fatty acids are a vital component of a bacterial cell, and especially important for inner and outer membrane structures <sup>443</sup>. My data suggested that even though the fatty acid component of the outer membrane appears to stimulate specific antibody production in the convalescent phase of infection the antibodies are functional and last until at least Month 3 post presentation. Siderophores are small ferric iron binding molecules secreted by microorganisms in response to iron stressing conditions <sup>429</sup>. As soon as entering the oxygen-rich environment (like peripheral blood), where ferrous iron is oxidized and turns into an insoluble form, microorganisms excrete siderophores to acquire and solubilize ferric iron <sup>429</sup>. In a pathogenic context, siderophores are a principal virulence factor that not only facilitate the infecting organism to grow but also disrupts iron homeostasis in the organelles of host cells <sup>429</sup>. The fact that genes involved in the siderophore manufacturing processes were specifically recognised by antibody in Day 8 plasma highlights the role of these structures during the early pathogenesis of *S. Typhi* in human blood. The window that *S. Typhi* is likely to be present in blood (an oxygen-rich environment) after successfully translocating through the barrier of intestinal ECs and replicating in macrophages is likely to be short and at least 48 hours (the time it takes to produce an antibody response) before the Day 8 sampling point.

Genes associated with the capsular polysaccharide Vi was only found in the gene pool produced after challenge with Day 8 plasma, while Vi polysaccharide vaccines confer protection for at least 3 years <sup>444</sup>. Therefore, to decipher the complete underlying mechanisms which foster the protective efficacy of the Vi polysaccharide vaccines requires more investigation.

A validation of the TraDIS data revealed that four antigens (excluding Vi polysaccharide) STY0796, STY1086, STY1372, and STY1498 and their equivalent antigens of *S. Paratyphi A* could induce antibody specific bactericidal activity, which stimulated strong and selective antibody-dependent complement-mediated killing. Additionally, bactericidal activity of anti Vi containing plasma to Vi+ and Vi- *S. Typhi* reconfirmed previous findings that prevention of Vi capsular to complement-mediated killing can be overcome by specific anti Vi antibody <sup>133</sup>. Further, the acquisition of natural antibody IgG against STY0796 (SPA1993), STY1086 (SPA1016), STY1372 (SPA1195) and especially STY1498 (SPA1306), in patients infected with *S. Typhi* and *S. Paratyphi A*, demonstrated these antigens are able to induce longitudinally specific antibody in both *S. Typhi* and *S. Paratyphi A* infections. Previously, I identified 3 *S. Typhi* antigens (STY0796, STY1086 and STY1372) that were promising for the serological diagnosis of typhoid (Chapter 4) <sup>333</sup>. STY1498, which encodes HlyE is an important *S. Typhi* virulence factor <sup>445–447</sup>. HlyE (also known as ClyA or SheA), which exists in both *S. Typhi* and *S. Paratyphi A*, is a haemolysin pore-forming toxin which accumulates in the periplasmic space of the bacterial membrane <sup>448</sup>. This protein was found to have diagnostic potential by immunoaffinity proteomic-based technology (IPT) <sup>413</sup> and on a protein array <sup>336</sup>. Further, its diagnostic discrimination has been evaluated among typhoid patients,

other infections, and healthy controls in children in Africa <sup>449</sup>. My data suggests that an immunogenic, inactivated form of HlyE may also act as a *S. Typhi*/*S. Paratyphi* A vaccine candidate.

The work in this chapter study would have been more sequential if all immunogenic antigens identified by the protein array and the TraDIS (Table 6.3) could have been validated and investigated for their ability to induce longitudinal IgG. Expanding the validation of the TraDIS results is current being performed in my research group and we aim to develop this work further into vaccinology. Further, a more expansive understanding of the antibody response to these antigens in different cohorts in different locations will be essential for understanding their utility and reproducibility in other settings.

In conclusion, the work presented in this chapter provides a unique set of data on *S. Typhi* antigens that can induce an antibody response and are capable of directing bactericidal killing during natural infections. Additionally, a subset of these antigens has been further validated *in vitro* and will add valuable information to the development of new generation of conjugate vaccines against enteric fever.

## **Chapter 7 Estimating the burden of typhoid carriers in a typhoid endemic city in Asia**

### **7.1 Introduction**

The principal agent of typhoid fever is *S. Typhi*, which has highly complex pathogenesis. Briefly, after being ingested, *S. Typhi* is translocated through the wall of the small intestine and disseminates in low concentrations throughout the body in the reticuloendothelial system via the blood, bone marrow, and liver. A further characteristic of *S. Typhi* is the ability of the organism to enter an asymptomatic carrier state, which is thought to be vital for geographical dispersal, maintenance of a diverse bacterial population, and disease transmission <sup>250</sup>. During asymptomatic carriage, organisms can be sustained for long periods (potentially decades) within the gallbladder or other internal organs/tissues (e.g. gallbladder and bone marrow) and are intermittently shed in faeces. Therefore, typhoid carriers, such as the notorious “Typhoid Mary” Mallon and Mr. N the Milker <sup>450</sup>, are assumed to be the longer-term reservoirs of infection, and carriers are thought to pose a substantial risk to public health <sup>250</sup>. Due to the stealth-like nature of typhoid carriage the mechanisms and precise epidemiological role of *S. Typhi* carriage in humans are poorly defined and the challenge of prospectively detecting carriers is a key objective for regional typhoid elimination.

Aiming to assess the frequency of *S. Typhi* carriers in a high transmission setting I investigated a cross-sectional population who underwent cholecystectomy in a general hospital in Kathmandu, Nepal <sup>15</sup>. This population had an *S. Typhi* carriage rate of 1.7%. I hypothesized that carriers have a unique immunological profile that

can be exploited to estimate the prevalence of carriage in a typhoid endemic population. To test this hypothesis, I identified carriage serological signatures using plasma samples from these individuals and evaluated these representative markers in plasma samples taken from the general population. This work provides a first-time serological method for estimating the prevalence of *S. Typhi* carriage in an endemic typhoid region.

## **7.2 Results**

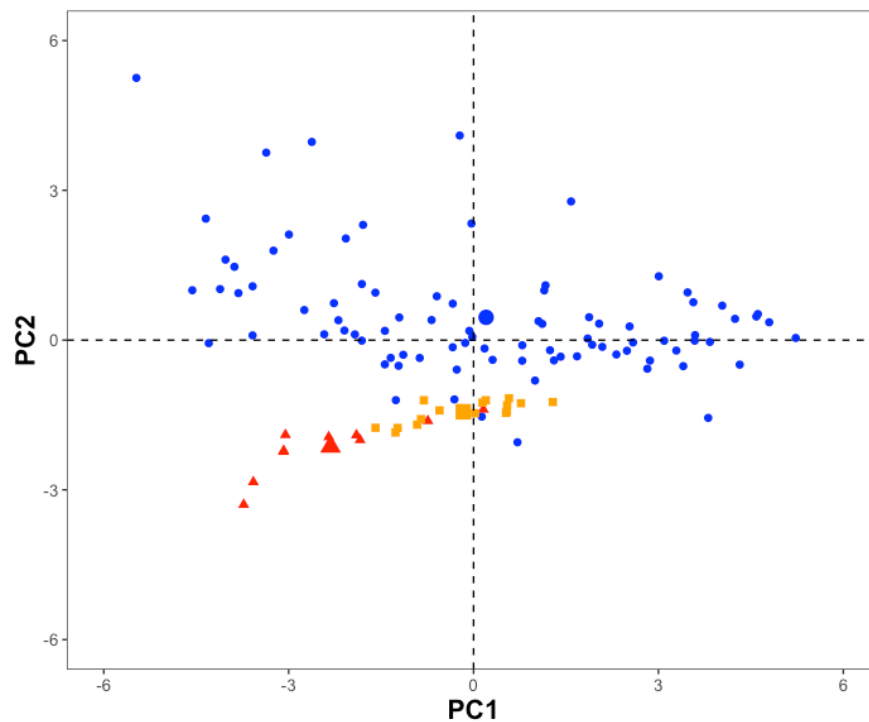
### **7.2.1 Typhoid carriers have a limited systemic inflammatory response**

Aiming to study the relation of the systemic inflammatory response to the state of *S. Typhi* asymptomatic carriage, I measured the concentration of eight pro-inflammatory cytokines (IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF- $\alpha$  and Interferon- $\gamma$ ) and two anti-inflammatory cytokines (IL-4 and IL-10) in plasma samples collected from patients undergoing cholecystectomy (*S. Typhi* positive, n=10; *S. Typhi* negative, n=17) and 89 acute typhoid patients (Day 8 after presentation). The entire data set of cytokine measurements were then subjected to a principal component analysis (PCA) to identify profiles associated with carriage. A visualization of these data identified discriminating profiles of these cytokines in acute typhoid patients, *S. Typhi* chronic carriers, and cholecystectomy controls (Figure 7.1).

The cytokine concentrations were subsequently examined to determine if the profiles were significantly discriminatory between patient groups. This analysis found that those with *S. Typhi* in their gallbladder had significantly lower concentrations of all assayed cytokines in comparison to those with acute typhoid

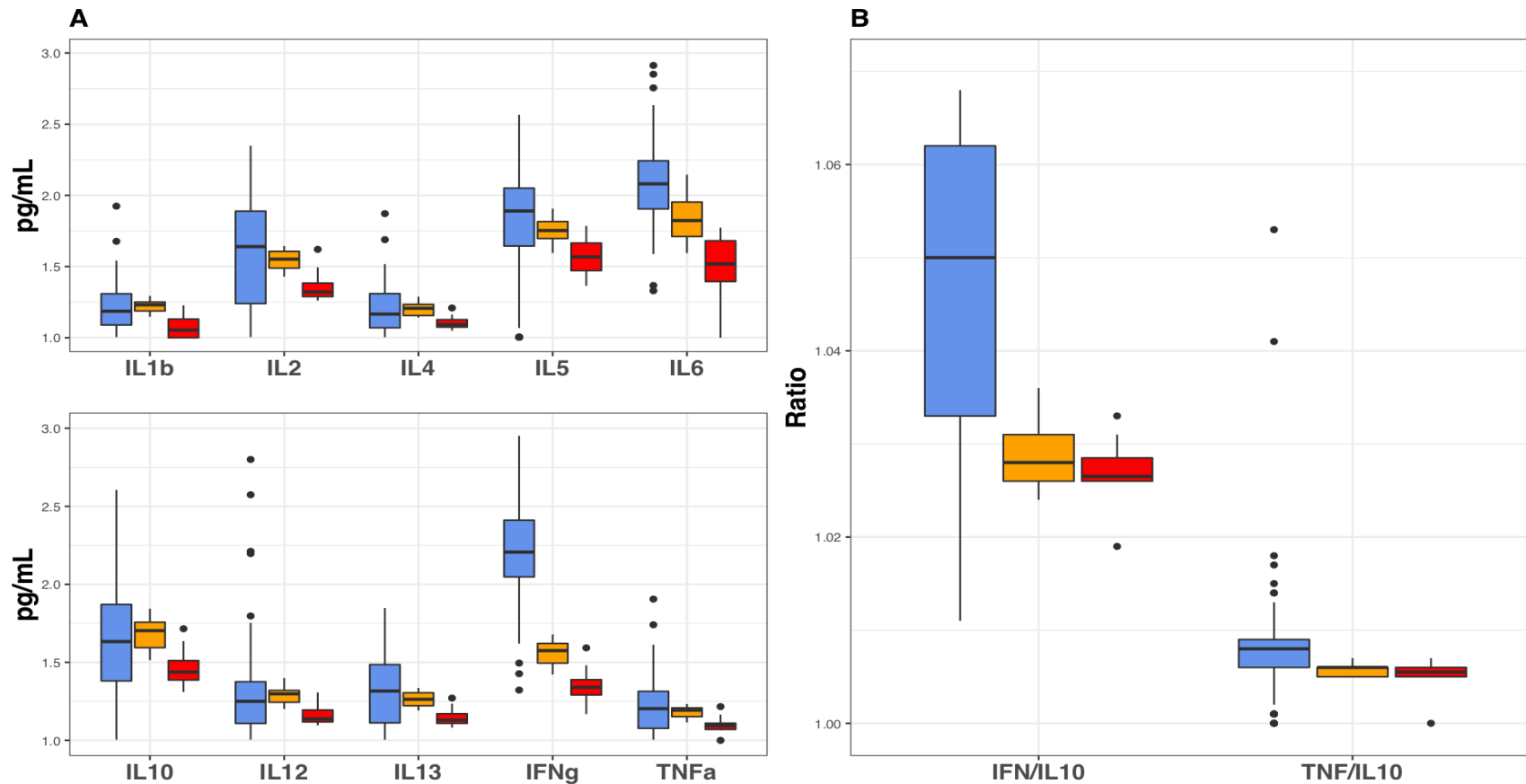
fever (day 8 after hospital admission) ( $p < 0.001$ , Wilcoxon signed-rank test), and the cholecystectomy controls ( $p < 0.001$ , Wilcoxon signed-rank test) (Figure 7.2).

Furthermore, I examined the ratio of INF- $\gamma$ :IL-10 and TNF- $\alpha$ :IL-10 among the three groups of patients to assess whether carriage was associated with a down regulation of the inflammatory response and found that the *S. Typhi* carriers had a significantly lower INF $\gamma$ :IL10 ratio than acute typhoid cases ( $p = 0.02$ , Wilcoxon signed-rank test).



**Figure 7.1** Visualization of cytokine profiles acquired from different groups of patients by Principal Component Analysis (PCA) plot

Cytokine measurements in plasma samples from different patient groups; acute typhoid (blue), cholecystectomy controls (orange) and *S. Typhi* carriers (red), were visualised by PCA plot. PC1, 2: Principle component 1, 2.



**Figure 7.2** Cytokine measurements in *Salmonella Typhi* carriers

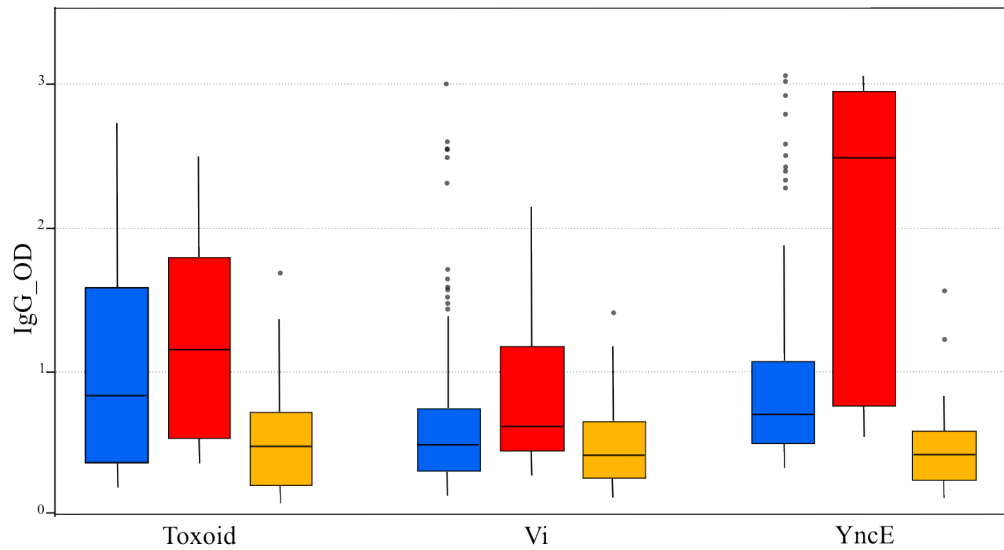
Boxplots showing cytokine measurements (pg/ml) in plasma from acute enteric fever patients (blue), cholecystectomy controls (orange), *S. Typhi* carriers (red). Every group of three boxes presents measurement of one pro-inflammatory/anti-inflammatory cytokine. Dark horizontal lines represent the mean cytokine measurement, with the box representing the 25<sup>th</sup> and 75<sup>th</sup> percentiles, whiskers represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles; outliers are represented by dots.



### 7.2.2 *Salmonella* Typhi carriers have a specific serological profile

Previous studies have shown that *S. Typhi* carriers elicit an elevated humoral immune response (i.e. higher IgG antibody titres in carriers compared to non-carriers) against the Vi antigen and STY1479, a hypothetical DNA binding protein encoded within the *S. Typhi* genome<sup>259,406</sup>. To validate these observations, I performed a series of ELISAs on the plasma samples taken from 148 patients with culture confirmed acute typhoid fever (day 8 after admission), 10 culture confirmed *S. Typhi* carriers, and 30 cholecystectomy controls to detect IgG against Vi (anti-Vi IgG) and STY1479 (anti-STY1479 IgG). I additionally measured circulatory IgG against additional 12 serodiagnostic proteins that I demonstrated to have utility in distinguishing between acute typhoid infections and asymptomatic controls<sup>340</sup>, and the recently described typhoid toxin<sup>137</sup>.

I found no differential IgG responses between *S. Typhi* carriers and cholecystectomy controls for the 12 serodiagnostic acute typhoid antigens ( $\rho > 0.05$ , Wilcoxon signed-rank test). Conversely, I found significantly elevated IgG against Vi ( $\rho = 0.05$ ), STY1479 ( $\rho = 0.008$ ), and typhoid toxin ( $\rho = 0.02$ , Wilcoxon signed-rank test) in *S. Typhi* carriers compared to the cholecystectomy controls (Figure 7.3).



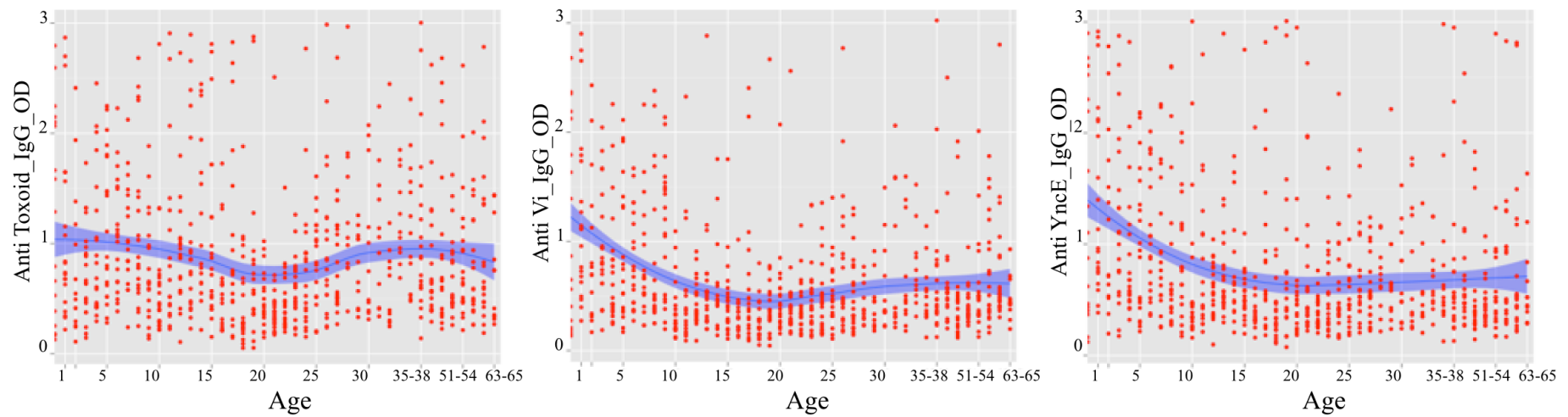
**Figure 7.3** IgG responses against different *Salmonella* Typhi antigens among patients with acute *Salmonella* Typhi infection, *Salmonella* Typhi carriers and cholecystectomy controls

Boxplots showing IgG measurements (OD) in plasma from acute *S. Typhi* infected patients (blue), *S. Typhi* carriers (red), cholecystectomy controls (orange). Every group of three boxplots presents measurement of IgG against one antigen. Dark horizontal lines represent the mean IgG measurement, with the box representing the 25<sup>th</sup> and 75<sup>th</sup> percentiles, whiskers represent the 5<sup>th</sup> and 95<sup>th</sup> percentiles; outliers are represented by dots.

### **7.2.3 Estimating the prevalence of *Salmonella* Typhi carriage in Kathmandu**

The fact that I could identify significantly elevated anti-Vi IgG, anti-STY1479 IgG, and anti-toxin IgG in *S. Typhi* carriers suggests that the humoral immune response against these antigens could be exploited to identify carriers in the community.

Therefore, I measured anti-Vi IgG, anti-STY1479 IgG, and anti-toxin IgG in an anonymous age-stratified (0-65years) collection of 733 plasma samples from asymptomatic individuals collected at the same study site and over the same time period as those undergoing cholecystectomies <sup>337</sup>. The raw data for the ELISAs from the general population are shown in Figure 7.4. These plots suggest some utility in using these antigens to detect carriers, as the majority had low antibody titres but there were a small number of outliers with highly elevated titres. The broad range of antibody titres in the population may have meant some overlap between the resulting antibody profiles and carriers.



**Figure 7.4** Acquisition of antibody IgG against typhoid toxoid, Vi and YncE antigens in an anonymous age-stratified (0-65 years) collection of plasma samples from Kathmandu

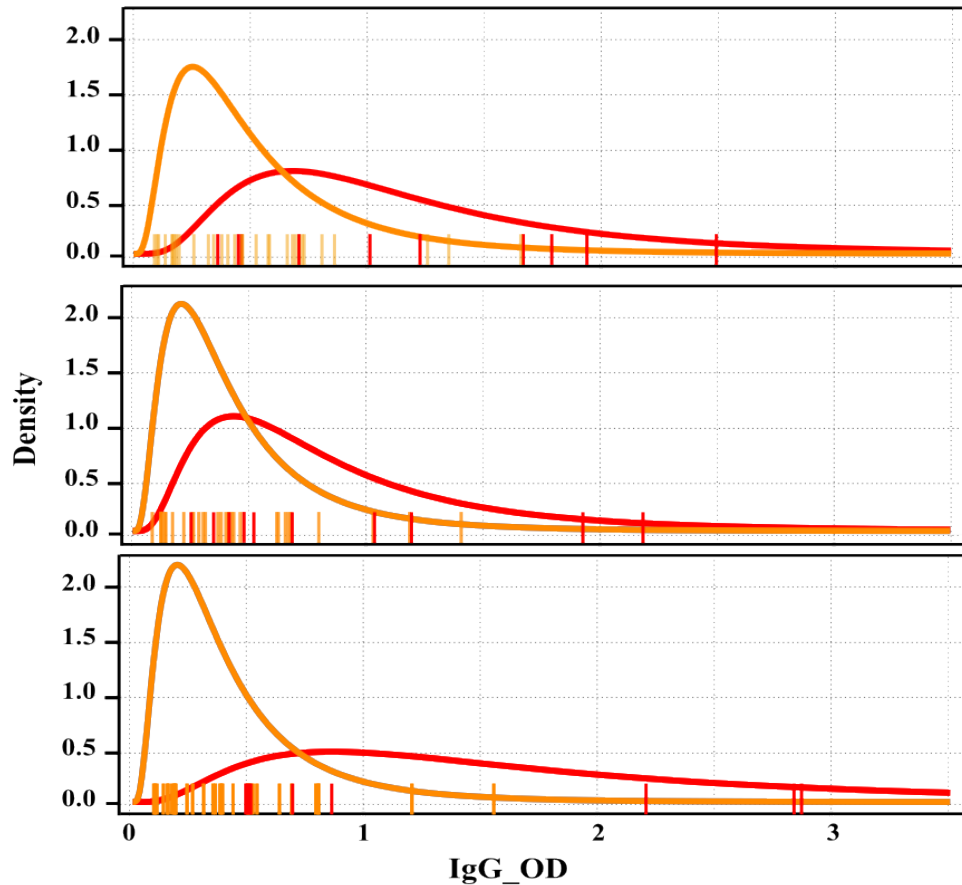
Scatter plots showing measurement of IgG (OD) against typhoid toxoid (leftmost panel), Vi (middle panel) and YncE (rightmost panel) in plasma samples collected from an age-stratified population of Kathmandu. Values (in OD) of antigen-induced antibodies are presented on Y-axes and ages are presented on X-axes. Solid lines represent age-dependent median of the IgG measurements with 95%CI presented by shaded areas.

Aiming to detect *S. Typhi* carriers, I investigated the set of data generated from *S. Typhi* carriers and cholecystectomy controls. The antibody responses exhibited positive correlation between antigens with  $\rho > 0.6$  ( $\rho < 0.05$ , Spearman's correlation coefficient test), which was also found in the set of data obtained from the asymptomatic individuals. There were, however, no precise cut-offs that could be used to perfectly differentiate potential carriers from non-carriers (the cholecystectomy controls). Therefore, I exploited log-normal model to estimate the density distribution against antibody titre for each of three antigens (based on the data from *S. Typhi* carriers and cholecystectomy controls) (Figure 7.5). The estimated values from these models were used to calculate the odds of being a *S. Typhi* carrier for each individual antigen. These odds were then used to calculate the combined/global odds of being a *S. Typhi* carrier based on three antigens and subsequently to construct a ROC curve (Figure 7.6).

Using this model, the best compromise between sensitivity and specificity (expected specificity of 1) corresponded to a threshold global odds of *S. Typhi* carriers of 4.2 (Figure 7.6), which estimated a prevalence of *S. Typhi* carriage, among the population of  $20 \leq \text{aged} \leq 65$  years, of 2.6% (95%CI=1.3%-4.4%). However, with the intention of targeting the elimination of carriers, the spectrum of identification of *S. Typhi* carriage was extended (while preserving high specificity of  $>0.9$ ), so that less cases of true *S. Typhi* carriage would be missed during the prediction process. The threshold odds were reduced to 2 and suggested that 14.1% (95%CI=10.7%-17.5%) of the population of  $20 \leq \text{aged} \leq 65$  years would need to be treated to better target elimination of carriers (Figure 7.6 and Figure 7.7).

Taking the best compromise between sensitivity and specificity (expected specificity of 1), the rate of 2.6% was used to extrapolate the prevalence of the total number of *S. Typhi*

carriers in Kathmandu, referencing proximal population figures from the 2011 government census (Table 2.8). Consequently, the estimated number of *S. Typhi* carriers in Kathmandu at this time (2011) was 27,623 (95%CI;13,811-46,748) existing among the population of  $20 \leq \text{aged} \leq 65$  years ( $n=1,062,458$ ).

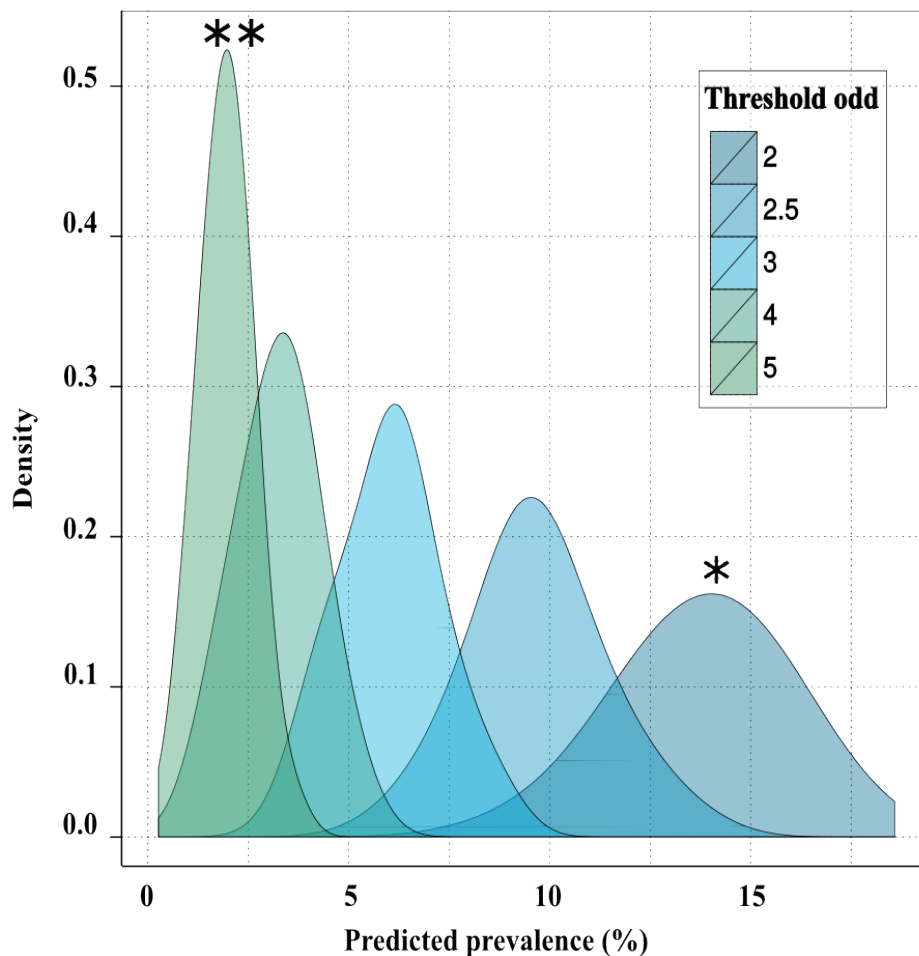


**Figure 7.5** Log-normal model for density distributions of antibody IgG titres against different antigens in *Salmonella* Typhi carriers and cholecystectomy controls.

Density distributions of antibody IgG against typhoid toxoid (top panel), Vi (middle panel), YncE (bottom panel) were estimated using a log-normal model. For each panel the red and orange curves represent the density distributions of *S. Typhi* carriers and cholecystectomy controls, respectively. Vertical red and orange bars represent the individual IgG measurements of 10 *S. Typhi* carriers and 30 cholecystectomy controls, respectively.







**Figure 7.7** The variation of predicted prevalence of *Salmonella* Typhi carriers in the population of  $20 \leq \text{aged} \leq 65$  years in the plasma bank

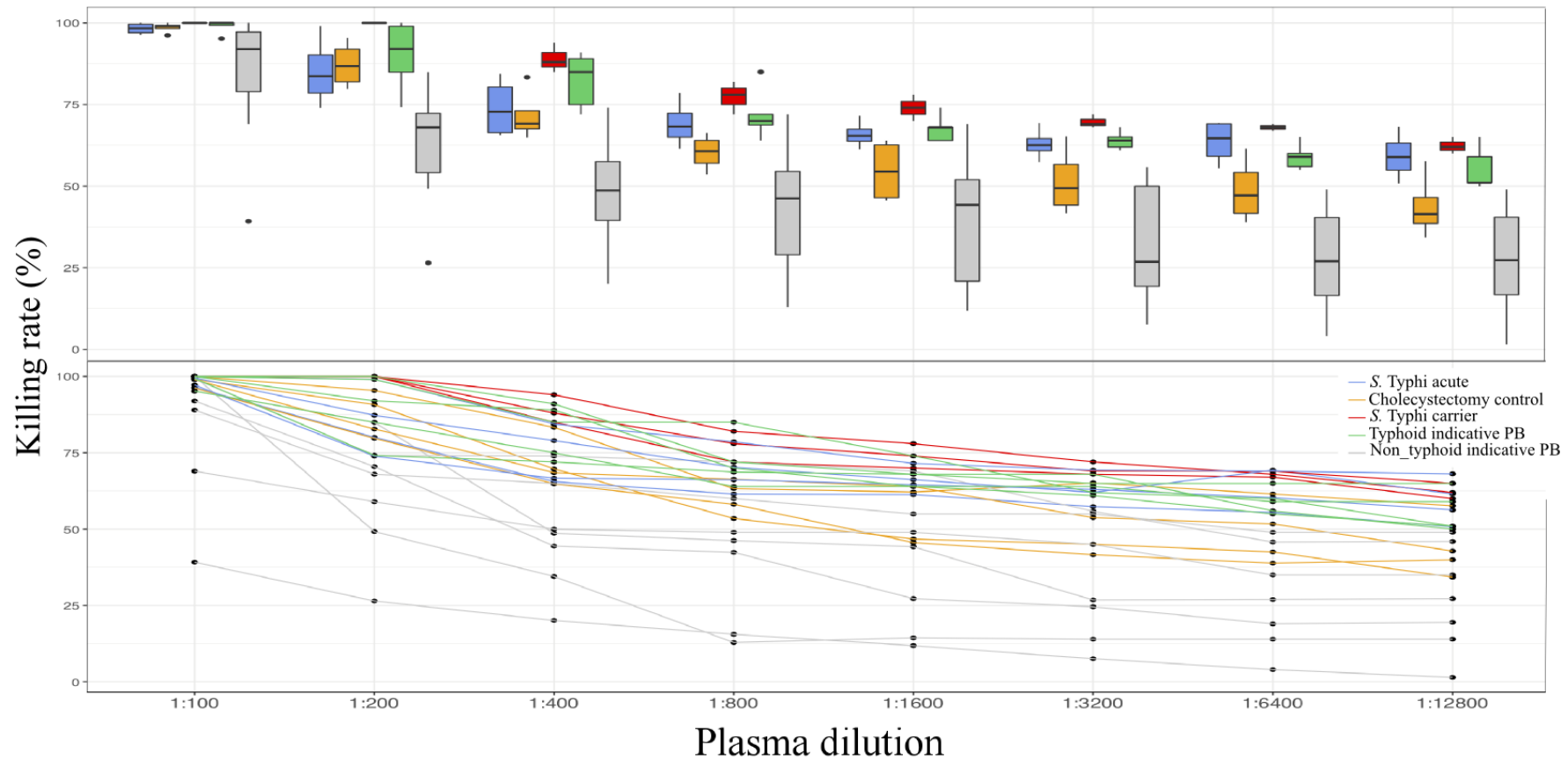
A chosen odd cut-off of being a *S. Typhi* carrier affects the sensitivity and specificity of the estimation and subsequently affects the predicted prevalence; the lower the odds cut-off the higher the predicted prevalence of *S. Typhi* carriers. The predicted prevalences (%) of *S. Typhi* carriers in the population of  $20 \leq \text{aged} \leq 65$  years in the plasma bank vary accordingly to the corresponding odds cut-offs. Colour represents the range of odds cut-offs, from 2 (dark blue) to 5 (light green). \* and \*\* are the odds cut-offs of 2 and 4.2, respectively, to which subsequently calculated the predicted prevalence of *S. Typhi* carrier of 14.1% and 2.6%, respectively, among the population of  $20 \leq \text{aged} \leq 65$  years in the plasma bank.

#### **7.2.4 Plasma from *Salmonella* Typhi carriers efficiently induce enhanced complement mediated killing**

Due to the anonymous nature of the population plasma bank we were unable to follow up any individuals with indicative antibody responses and confirm the carriage and/or excretion of *S. Typhi*. To address this limitation, I performed a series of functional antibody assays, measuring the ability of plasma from *S. Typhi* carriers to induce complement mediated bactericidal activity in comparison to plasma from acute typhoid patients and the cholecystectomy controls.

At any plasma dilution (of 2-fold serial dilution started from 1:100 to 1:12,800) at 1.5 hours post inoculation, the plasma from the *S. Typhi* carriers exhibited significantly greater bactericidal activity than plasma from the acute typhoid patients and the cholecystectomy controls ( $p < 0.001$ , Wilcoxon signed-rank test) (Figure 7.8). These data indicate that *S. Typhi* carriage and IgG responses against Vi, STY1479, and typhoid toxoid correlate with a functional antibody response, and may be an additional marker of carriage associated immunity. Furthermore, samples from the plasma bank with an antibody response indicative of carriage exhibited substantially greater bactericidal activity than plasma from those with low IgG responses against the three informative antigens ( $p = 0.01$ , Wilcoxon signed-rank test). Plasma from the predicted *S. Typhi* carriers induced greater bactericidal activity than the acute typhoid plasma samples yet exhibited comparable activity to plasma from the *S. Typhi* carriers. These data confirm the functionality of antibody in those with an IgG profile indicative of carriage, identified by antibody IgG profile

against to these three antigens and again imply carriage associated immunity in this population.



**Figure 7.8** Antibody-dependent complement mediated bactericidal killing of *Salmonella Typhi*

Boxplots (top panel) present killing rates of the assayed plasma samples (across 2-fold serial dilutions, started from 1:100 to 1:12,800), which were collected from acute *S. Typhi* infected patients (blue), cholecystectomy controls (orange), *S. Typhi* carriage (red), typhoid indicative plasma bank (green) and non-typhoid indicative plasma bank (grey). Lines (bottom panel) show individual killing rates of the same set of data.

### 7.3 Discussion

During invasive *Salmonella* infection, PAMPs (pathogen-associated-molecular-patterns) and DAMPs (danger-associated molecular-patterns) are recognised by PRRs (pattern recognition receptors)<sup>99</sup>, which activates the innate immune system inducing the recruitment of immune cells such as neutrophils and macrophages and the generation of pro-inflammatory cytokines<sup>451 99,230,452–454</sup>. Cytokines contribute greatly to both pathogenesis and control of systemic *Salmonella* infections<sup>230,452</sup>. Elevated production of pro-inflammatory cytokines, which promotes a systemic inflammatory response, have been observed in patients with acute *S. Typhi* infections<sup>230,452,455</sup> and interferons (IFNs) are the signatures for immunological responses to *Salmonella* infection<sup>99,192</sup>. However, limited data has examined the cytokine profile of individuals with chronic typhoid carriers. Aiming to understand if typhoid chronic carriages induce a comparable cytokine profile as those with acute infections, I investigated a panel of pro-inflammatory cytokines in plasma samples from acute typhoid patients and chronic carriers. Using principal component analysis to visualise the cytokine profiles obtained from different groups of patients, I was able to show that cytokine profiles are different between the various phases of *S. Typhi* infections (acute or chronic) and from controls. The subsequent analysis revealed distinguishable cytokines profiles in plasma from different patient groups, showing significant lower cytokine concentrations in plasma samples from chronic carriages than those from acute typhoid patients. The consistent pattern between all examined pro-inflammatory cytokines suggests that *S. Typhi* does not stimulate as strong a systemic pro-inflammatory response during chronic carriage stage as it does during an acute infection. Additionally, the level of systemic pro-inflammatory cytokines in chronic carriage was found to be significantly lower than those in the

cholecystectomy controls. These observations indicate that individuals with *S. Typhi* in their gallbladder had diminished concentrations of pro-inflammatory cytokines. Additionally, the ratio INF- $\gamma$ :IL-10 was significantly lower in the *S. Typhi* chronic carriers in comparison to the acute patients and cholecystectomy controls. IL-10 inhibits synthesis of the pro-inflammatory cytokines IFN- $\gamma$ , IL-2, IL-3 and TNF $\alpha$ , down regulating the inflammatory response and macrophage activation <sup>456</sup>. These data suggest that *S. Typhi* in the gallbladder is actively suppressing the inflammatory response. The Vi capsule is known to be immunomodulatory <sup>173,183</sup> and the anti-inflammatory effect of IL-10 may facilitate the distinct profile of pro-inflammatory cytokines and suppress the development of the febrile disease in chronic carriers.

*Salmonella* carriage is of public concern as these people are a persistent reservoir of infection, contributing to disease transmission and ultimately to new disease episodes in the community <sup>250</sup>. Additionally, *S. Typhi* carriers have a significantly increased risks of gallbladder carcinoma as a consequence of the chronic irritation of their gallbladder <sup>254,255</sup>. Therefore, the prospective detection of chronic *Salmonella* carriers is essential for eliminating local enteric fever transmission <sup>257</sup> as well as being valuable knowledge for those carrying the pathogen or having gallbladder abnormalities <sup>254</sup>. Yet, detection of enteric chronic carriers is not straight forward because they are outwardly asymptomatic.

Serological examination, especially the utility of anti-Vi antibody <sup>200,259,457,458</sup>, is a practical approach to identify *S. Typhi* chronic carriers <sup>250,257,258</sup>. However, there are drawbacks to the use of anti-Vi antibody in typhoid endemic areas as elevated anti-Vi antibody in healthy individuals is highly prevalent in these regions <sup>457</sup>.

Campaigns with Vi vaccine in typhoid endemic areas <sup>459</sup> further compound the situation. My data demonstrated that antibody (IgG) against YncE, typhoid toxin, and Vi are significantly differential between *S. Typhi* chronic carriers, acute typhoid cases and cholecystectomy controls. I used these IgG profiles to detect *S. Typhi* chronic carriers among individuals of  $20 \leq \text{aged} \leq 65$  years in a plasma bank in Kathmandu, a typhoid endemic area. By combining these antibody profiles, I estimated an upper limited prevalence of *S. Typhi* carriage of 14.1% with the least missing cases and a specificity of  $>0.9$ . However, in order to not misdiagnose any non-*S. Typhi* carriers, the rate was predicted to be 2.6% with highest specificity ( $=1$ ). This estimation of 2.6% is comparable with the findings from the definite diagnosis (culture confirmed) of the organism *S. Typhi* in gallbladder of those undergoing cholecystectomy reported in this original study (1.7% mixed sex) <sup>15</sup>, and previous studies (4% for female and 2.9% for male) <sup>460</sup>. I then calculated that there were an estimated 27,623 (95%CI;13,811-46,748) carriers existing among the population of  $20 \leq \text{aged} \leq 65$  in year 2011, in Kathmandu valley, Nepal. This work, for the first time in almost 4 decades since the estimation of the number of *S. Typhi* carriage among Chilean population reported by Levine et. al in 1982 <sup>461</sup>, delivers a method to estimate the number and prevalence of *S. Typhi* chronic carrier in an endemic area.

Due to the anonymous nature of the population plasma bank, the confirmation of *S. Typhi* chronic carriage based on serology was not possible. However, I further found an association between the bactericidal activity and IgG profiles in plasma from *S. Typhi* carriers. I additionally found that samples in the plasma bank that had an antibody profile indicative of carriage also exhibited a comparable bactericidal activity. This observation provides supporting data that these individuals are likely

true carriers. Of note, the data presented in Chapter 6 suggested that anti-YncE (STY1479) antibody did not induce antibody-dependent complement-mediated bactericidal activity. Consequently, anti-YncE antibody was not responsible for the high level of bactericidal activity against *S. Typhi* in plasma of *S. Typhi* carriers.

Even though the estimated rate of *S. Typhi* carriers was highly comparable with previous studies, the method used to identify *S. Typhi* carriage was developed on a small sample size and may have been under powered. Consequently, I suggest these methods are validated in a large sample size and in different endemic settings.

In conclusion, *S. Typhi* carriers have a unique immunological profile with a distinct cytokine pattern and antigen specific antibody profiles in their plasma. My findings delivered new insights into methods that can be used for identifying and estimating the prevalence of *S. Typhi* carriage in an endemic typhoid population.



## Chapter 8 General discussion

As we move into the era of potential typhoid elimination through the use of conjugate vaccines we need better tools to determine disease burden. We need better point of care tests to attribute appropriate antimicrobial treatment and to measure the potential impact of immunization or alternative interventions. Also, the detection of typhoid carriers is also essential to ensure the disease is eliminated rapidly and to limit sustained transmission from carriers after acute shedding is reduced by immunization. My thesis outlines several routes that may contribute to these areas.

The emergence of MDR and XDR isolates of *S. Typhi* <sup>462</sup> and an increasing incidence of *S. Paratyphi A* are contributing to changing the international dynamics of enteric fever <sup>463</sup>. These changes add urgency to the demand for more efficient enteric fever control campaigns. My investigation into the key factors associated with typhoid elimination in Vietnam demonstrated that national economic growth, affording improved quality of drinking water, and better sanitation was the greatest contribution to disease control. These factors reduce the general exposure of humans to typhoid causing organisms. Consequently, Vietnam should be considered as an exemplar to other current endemic typhoid areas such as Kathmandu (Nepal) and Chittagong (Bangladesh). However, these conditions may not be promptly achieved in these areas. Therefore, suitable interim measures are required to better manage the disease and measure disease burden, the more accurate diagnosis of acute and carriage cases is essential. Various approaches have been investigated to overcome the limitations of the available diagnostic tests for acute typhoid. Specifically, most approaches have low sensitivity (bacterial culture) or modest specificity (serology

tests using somatic and flagellar antigens); therefore, these methods are unlikely to impact on the development of simple, rapid diagnostic tests over the coming years. My study evaluated a range of *S. Typhi* specific antigens for the purpose of identifying acute typhoid cases amongst other febrile infections and afebrile controls in Bangladesh. The generated data signified that the combination of the traditional *S. Typhi* capsular antigen Vi with at least one specific *S. Typhi* protein antigen may provide some serological utility for typhoid diagnostics. These antigens may aid in the issue of diagnosing febrile disease of unknown origin and facilitate the use of appropriate antimicrobial therapy. Further, my study also aimed to address the challenges of prospectively detecting asymptomatic typhoid carriers, which is strategic for regional typhoid elimination. My findings delivered unprecedented advances for identifying and estimating the prevalence of *S. Typhi* carriage in human populations. Another crucial aim for the rational control of enteric fever is widespread immunization campaigns of a new generation of vaccines which are safe and confer protection to both variants of typhoidal *Salmonella*. Correspondingly, my study also focused on exploring the longitudinal host immune responses against *S. Typhi* and *S. Paratyphi A*, which delivered a unique set of data regarding a panel of novel immunogenic antigens that are shared between the two organisms during natural infection. These findings provide valuable preliminary data that may be applied to the development of a new generation of conjugate vaccines against enteric fever. Additionally, to control enteric fever in endemic areas where organisms that exhibit resistance to the last resort antimicrobials may require alternative treatment approaches. My work additionally investigated the antibody response against typhoid toxin, specifically regarding the acquisition of antibody during natural *S. Typhi* and *S. Paratyphi A* infection and toxin neutralisation capability. This

informative data provides supportive evidence which may provide a significant step towards the development of monoclonal anti-toxin antibody that could be used for enteric fever therapy.

During the work in this thesis I was granted a UK patent for the use of various protein antigens for the diagnosis of typhoid and we have since licensed these antigens to Becton-Dickenson for use in their multi-pathogen tropical febrile disease screen (Malaria, dengue, and typhoid fever). I see the development of multi-pathogen assays the most relevant way forward for diagnosing febrile disease in the tropical and the differentiation of bacterial, viral, or parasitic disease should be a major advance. A further utility of my antigens could be to measure disease burden in areas under consideration for immunization programmes. The only current surveillance data arises from Widal test or blood culture, both of which have limitations. My data suggests that we can measure sero-conversion on populations that have typhoid fever. Consequently, these data may be used to assess where typhoid vaccines could have the greatest impact. This would be a hugely beneficial tool for GAVI eligible countries and may be supported by the WHO. Furthermore, the standardisation of such a test(s) would allow for a comparatively cost-effective method to assess the impact of vaccination longitudinally. Again, we do not have such tools and my data implies that such approaches have potential.

The findings described in my thesis required further assessment through comparable studies in additional cohorts. Additionally, further research is currently being conducted in my laboratory and other research groups in Asia to validate the finding regarding some of the *S. Typhi* antigens described in my thesis; specifically, with

respect to their ability to induce acute and longitudinal immune responses in different patient populations. These studies will be crucial for future development of commercial enteric fever diagnostic tests and for conjugate vaccine development. In conclusion, my thesis contributes to the data required to assist in rational enteric fever control campaigns in endemic areas by providing new fundamental, valuable data for the purposes of diagnostics, vaccines and potential new treatments for enteric fever. Ultimately, I hope this work can have a direct impact on the future policy of typhoid disease control in endemic and non-endemic regions.

## Chapter 9 References

1. Papagrigorakis, M. J., Yapijakis, C., Synodinos, P. N. & Baziotopoulou-Valavani, E. DNA examination of ancient dental pulp incriminates typhoid fever as a probable cause of the Plague of Athens. *Int. J. Infect. Dis.* **10**, 206–214 (2006).
2. Adler, R. & Mara, E. *Typhoid fever: a history*. (McFarland, 2016). doi:978-1-4766-2209-5
3. Parry, M. C. Epidemiological and clinical aspects of human typhoid fever. in *Salmonella Infections \_ Clinical, Immunological and Molecular Aspects* (eds. Mastroeni, P. & Maskell, D.) 1–24 (Cambridge University Press, 2006).
4. Maskey, A. P. *et al.* Salmonella enterica serovar Paratyphi A and S. enterica serovar Typhi cause indistinguishable clinical syndromes in Kathmandu, Nepal. *Clin. Infect. Dis.* **42**, 1247–53 (2006).
5. Parkhill\*, J. *et al.* Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. *Nature* **413**, 199–228 (2001).
6. McClelland, M. *et al.* Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of Salmonella enterica that cause typhoid. *Nat. Genet.* **36**, 1268–1274 (2004).
7. Hiyoshi, H., Tiffany, C. R., Bronner, D. N. & Andreas, J. B. Typhoidal Salmonella serovars : ecological opportunity and the evolution of a new pathovar. 527–541 (2018). doi:10.1093/femsre/fuy024
8. Karkey, A. *et al.* The Ecological Dynamics of Fecal Contamination and Salmonella Typhi and Salmonella Paratyphi A in Municipal Kathmandu Drinking Water. *PLoS Negl. Trop. Dis.* **10**, e0004346 (2016).
9. Ochiai, R. L. *et al.* A study of typhoid fever in five Asian countries : disease burden and implications for controls. *Bull. World Health Organ.* **039818**, (2008).
10. Parry, C. M., Hien, T. T., Dougan, G., White, N. J. & Farrar, J. J. Typhoid fever. *N. Engl. J. Med.* **347**, 1770–1782 (2002).
11. von Kalckreuth, V. *et al.* The Typhoid Fever Surveillance in Africa Program (TSAP): Clinical, Diagnostic, and Epidemiological Methodologies. *Clin. Infect. Dis.* **62 Suppl 1**, S9–

S16 (2016).

12. Brenner, F. W., Villar, R. G., Angulo, F. J., Tauxe, R. & Swaminathan, B. Salmonella Nomenclature. *J. Clin. Microbiol.* **38**, 2465–2467 (2000).
13. Parry, C. M. Typhoid fever. *Curr. Infect. Dis. Rep.* **6**, 27–33 (2004).
14. Santos, R. L. *et al.* Animal models of Salmonella infections : enteritis versus typhoid fever. *Microbes Infect.* **3**, 1335–1344 (2001).
15. Dongol, S. *et al.* The microbiological and clinical characteristics of invasive salmonella in gallbladders from cholecystectomy patients in kathmandu, Nepal. *PLoS One* **7**, e47342 (2012).
16. Crump, J. A., Gordon, M. A. & Parry, C. M. Epidemiology, Clinical Presentation, Laboratory Diagnosis, Antimicrobial Resistance, and Antimicrobial Management of Invasive Salmonella Infections. *Journals.ASM.* **28**, 901–937 (2015).
17. Kidgell, C. *et al.* Salmonella typhi , the causative agent of typhoid fever, is approximately 50,000 years old. *Science (80-. ).* **2**, 39–45 (2002).
18. Roumagnac, P. *et al.* Evolutionary History of Salmonella Typhi. *Science (80-. ).* **314**, 1301–1304 (2006).
19. Dunstan, S. J. *et al.* Genes of the Class II and Class III Major Histocompatibility Complex Are Associated with Typhoid Fever in Vietnam. *J. Infect. Dis.* **183**, 261–268 (2001).
20. Arya, S. C. & Agarwal, N. Host factors and typhoid fever-associated intestinal perforation in children in Nigeria. *Pediatr. Surg. Int.* **28**, 753 (2012).
21. van de Vosse, E. *et al.* Susceptibility to typhoid fever is associated with a polymorphism in the cystic fibrosis transmembrane conductance regulator (CFTR). *Hum. Genet.* **118**, 138–140 (2005).
22. Butler, T., Islam, A., Kabir, I. & Jones, P. K. Patterns of Morbidity and Mortality in Typhoid Fever Dependent on Age and Gender: Review of 552 Hospitalized Patients with Diarrhea. *Rev. Infect. Dis.* **13**, 85–90 (1991).
23. Parry, C. M. *et al.* Risk factors for the development of severe typhoid fever in Vietnam. *BMC Infect. Dis.* **14**, 73 (2014).
24. Bhan, M. K. *et al.* Association between Helicobacter pylori Infection and Increased Risk of Typhoid Fever. *J. Infect. Dis.* **186**, 1857–1860 (2002).

25. Ali, G., Rashid, S., Kamli, M. a, Shah, P. a & Allaqaband, G. Q. Spectrum of neuropsychiatric complications in 791 cases of typhoid fever. *Trop. Med. Int. Health* **2**, 314–8 (1997).
26. Lutterloh, E. *et al.* Multidrug-resistant typhoid fever with neurologic findings on the Malawi-Mozambique border. *Clin. Infect. Dis.* **54**, 1100–1106 (2012).
27. Sejvar, J. *et al.* Neurologic Manifestations Associated with an Outbreak of Typhoid Fever, Malawi - Mozambique, 2009: An Epidemiologic Investigation. *PLoS One* **7**, (2012).
28. Szabo, I. *et al.* Neurologic complications and sequellae of infectious diseases in Uganda and Kenya: Analysis of 288 cases from two rural hospitals. *Neuro Endocrinol. Lett.* **34**, 28–31 (2013).
29. Wadia, R. S., Ichaporia, N. R., Kiwalkar, R. S., Amin, R. B. & Sardesai, H. V. Cerebellar ataxia in enteric fever. *J. Neurol. Neurosurg. Psychiatry* **48**, 695–697 (1985).
30. Hosoglu, S. *et al.* Risk factors for enteric perforation in patients with typhoid fever. *Am. J. Epidemiol.* **160**, 46–50 (2004).
31. Van Den Bergh, E. T. A. M., Hussein Gasem, M., Keuter, M. & Dolmans, M. V. Outcome in three groups of patients with typhoid fever in Indonesia between 1948 and 1990. *Trop. Med. Int. Heal.* **4**, 211–215 (1999).
32. Bhutta, Z. A. Impact of age and drug resistance on mortality in typhoid fever. *Arch. Dis. Child.* **75**, 214–217 (1996).
33. Parry, C. M. *et al.* Randomized Controlled Comparison of Ofloxacin, Azithromycin, and an Ofloxacin-Azithromycin Combination for Treatment of Multidrug-Resistant and Nalidixic Acid-Resistant Typhoid Fever. *Antimicrob. Agents Chemother.* **51**, 819 LP-825 (2007).
34. Wain, J. *et al.* Molecular typing of multiple-antibiotic-resistant *Salmonella enterica* serovar Typhi from Vietnam: application to acute and relapse cases of typhoid fever. *J. Clin. Microbiol.* **37**, 2466–2472 (1999).
35. Tran, T. H. *et al.* Short course of ofloxacin for treatment of multidrug-resistant typhoid. *Clin. Infect. Dis.* **20**, 917–923 (1995).
36. Yew, F. S., Chew, S. K., Goh, K. T., Monteiro, E. H. & Lim, Y. S. Typhoid fever in Singapore: a review of 370 cases. *J. Trop. Med. Hyg.* **94**, 352–357 (1991).
37. Gotuzzo, E. *et al.* Association between specific plasmids and relapse in typhoid fever. *J. Clin.*

- Microbiol.* **25**, 1779 LP-1781 (1987).
38. Butler, T., Rumans, L. & Arnold, K. Response of typhoid fever caused by chloramphenicol-susceptible and chloramphenicol-resistant strains of *Salmonella typhi* to treatment with trimethoprim-sulfamethoxazole. *Rev. Infect. Dis.* **4**, 551–561 (1982).
  39. Wain, J. *et al.* Molecular typing of multiple-antibiotic-resistant *Salmonella enterica* serovar Typhi from Vietnam: application to acute and relapse cases of typhoid fever. *J. Clin. Microbiol.* **37**, 2466–72 (1999).
  40. Sinha, A. *et al.* Typhoid fever in children aged less than 5 years. *Lancet* **354**, 734–737 (1999).
  41. Lin, F. Y. C. *et al.* The epidemiology of typhoid fever in the Dong Thap Province, Mekong Delta region of Vietnam. *Am. J. Trop. Med. Hyg.* **62**, 644–648 (2000).
  42. Butler, T. Treatment of typhoid fever in the 21st century : promises and shortcomings. (2011).
  43. Yanagi, D. *et al.* Emergence of fluoroquinolone-resistant strains of *Salmonella enterica* in Surabaya, Indonesia. *Diagn. Microbiol. Infect. Dis.* **64**, 422–426 (2009).
  44. Lutterloh, E. *et al.* Multidrug-resistant typhoid fever with neurologic findings on the Malawi-Mozambique border. *Clin. Infect. Dis.* **54**, 1100–6 (2012).
  45. Menezes, G. A., Harish, B. N., Khan, M. A., Goessens, W. H. F. & Hays, J. P. Antimicrobial resistance trends in blood culture positive *Salmonella Typhi* isolates from Pondicherry, India, 2005-2009. *Clin. Microbiol. Infect.* **18**, 239–245 (2012).
  46. Shirakawa, T. *et al.* Decreased susceptibility to fluoroquinolones and *gyrA* gene mutation in the *Salmonella enterica* serovar Typhi and Paratyphi A isolated in Katmandu, Nepal, in 2003. *Diagn. Microbiol. Infect. Dis.* **54**, 299–303 (2006).
  47. Baker, S., Thomson, N., Weill, F.-X. & Holt, K. E. Genomic insights into the emergence and spread of antimicrobial-resistant bacterial pathogens. *Science (80-. ).* **360**, 733–738 (2018).
  48. Kaurthe, J. Increasing antimicrobial resistance and narrowing therapeutics in typhoidal salmonellae. *J. Clin. Diagn. Res.* **7**, 576–9 (2013).
  49. Anderson, E. S. The problem and implications of chloramphenicol resistance in the typhoid bacillus. *J. Hyg. (Lond).* **74**, 289–299 (1975).
  50. Rowe, B., Ward, L. R. & Threlfall, E. J. Multidrug-Resistant *Salmonella typhi* : A Worldwide Epidemic. *Clin. Infectious Dis.* **24**, S106–S109 (1997).



51. Zaki, S. A. & Karande, S. Multidrug-resistant typhoid fever: a review. *J. Infect. Dev. Ctries.* **5**, 324–337 (2011).
52. Chinh, N. T. *et al.* A randomized controlled comparison of azithromycin and ofloxacin for treatment of multidrug-resistant or nalidixic acid-resistant enteric fever. *Antimicrob. Agents Chemother.* **44**, 1855–9 (2000).
53. Threlfall, E. J., Ward, L. R., Skinner, J. A., Smith, H. R. & Lacey, S. Ciprofloxacin-resistant *Salmonella typhi* and treatment failure. *Lancet (London, England)* **353**, 1590–1591 (1999).
54. Crump, J. A. *et al.* Clinical response and outcome of infection with *Salmonella enterica* serotype typhi with decreased susceptibility to fluoroquinolones: A United States FoodNet multicenter retrospective cohort study. *Antimicrob. Agents Chemother.* **52**, 1278–1284 (2008).
55. Effa, E. E. & Bukirwa, H. Azithromycin for treating uncomplicated typhoid and paratyphoid fever (enteric fever). *Cochrane Database Syst Rev* **4**, (2008).
56. Girgis, N. I., Tribble, D. R., Sultan, Y. & Farid, Z. Short course chemotherapy with cefixime in children with multidrug-resistant *Salmonella typhi* Septicaemia. *J. Trop. Pediatr.* **41**, 364–365 (1995).
57. Soe, G. B. & Overturf, G. D. Treatment of typhoid fever and other systemic salmonellosis with cefotaxime, ceftriaxone, cefoperazone, and other newer cephalosporins. *Rev. Infect. Dis.* **9**, 719–736 (1987).
58. Pape, J. W., Gerdes, H., Oriol, L. & Johnson, W. D. J. Typhoid fever: successful therapy with cefoperazone. *J. Infect. Dis.* **153**, 272–276 (1986).
59. Islam, A., Butler, T., Kabir, I. & Alam, N. H. Treatment of typhoid fever with ceftriaxone for 5 days or chloramphenicol for 14 days: a randomized clinical trial. *Antimicrob. Agents Chemother.* **37**, 1572–1575 (1993).
60. Dutta, P. *et al.* Ceftriaxone therapy in ciprofloxacin treatment failure typhoid fever in children. *Indian J. Med. Res.* **113**, 210–213 (2001).
61. Frenck, R. W. J. *et al.* Short-course azithromycin for the treatment of uncomplicated typhoid fever in children and adolescents. *Clin. Infect. Dis.* **38**, 951–957 (2004).
62. Ferreccio, C. *et al.* Efficacy of ciprofloxacin in the treatment of chronic typhoid carriers. *J. Infect. Dis.* **157**, 1235–1239 (1988).

63. Gotuzzo, E. *et al.* Use of norfloxacin to treat chronic typhoid carriers. *J. Infect. Dis.* **157**, 1221–1225 (1988).
64. CM, N., PC, W. & Jr. Treatment of typhoid carriers with amoxicillin: Correlates of successful therapy. *JAMA* **239**, 2352–2354 (1978).
65. J Clementi, K. *Treatment of Salmonella carriers with trimethoprim sulfamethoxazole. Canadian Medical Association journal* **112**, (1975).
66. WE, P. Treatment of chronic typhoid carriers with ampicillin. *JAMA* **217**, 913–915 (1971).
67. Buckle, G. C., Walker, C. L. F. & Black, R. E. Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010. *J. Glob. Health* **2**, 10401 (2012).
68. Ochiai, R. L. *et al.* Salmonella Paratyphi A Rates, Asia. *Emerg. Infect. Dis.* 0–2 (2005).
69. Fangtham, M., Wilde, H. & Asia, C. Emergence of Salmonella paratyphi A as a Major Cause of Enteric Fever: Need for Early Detection, Preventive Measures, and Effective Vaccines. *Int. Soc. Travel Med.* **15**, 344–350 (2008).
70. Arndt, M. B. *et al.* Estimating the Burden of Paratyphoid A in Asia and Africa. *PLoS Negl. Trop. Dis.* **8**, (2014).
71. Woods, C. W. *et al.* Emergence of Salmonella enterica serotype Paratyphi A as a major cause of enteric fever in Kathmandu, Nepal. *Trans. R. Soc. Trop. Med. Hyg.* **100**, 1063–1067 (2006).
72. Gupta, V., Kaur, J. & Chander, J. An increase in enteric fever cases due to Salmonella Paratyphi A in & around Chandigarh. *Indian J. Med. Res.* **129**, 95–98 (2009).
73. Maskey, A. P. *et al.* Emerging trends in enteric fever in Nepal: 9124 cases confirmed by blood culture 1993–2003. *Trans. R. Soc. Trop. Med. Hyg.* **102**, 91–95 (2008).
74. Wain, J. *et al.* Specimens and culture media for the laboratory diagnosis of typhoid fever. *J. Infect. Dev. Ctries.* **2**, (2008).
75. Crump, J. a *et al.* The global burden of typhoid fever. *Bull. World Health Organ.* **82**, 346–53 (2004).
76. Mogasale, V. *et al.* Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment. *Lancet. Glob. Heal.* **2**, e570–80 (2014).

77. Stanaway, J. D. The global burden of typhoid and paratyphoid fevers : a systematic analysis for the Global Burden of Disease Study 2017. *Lancet* **3099**, 30685–6 (2019).
78. Ackers, M., ND, P., RV, T. & ED, M. Laboratory-based surveillance of salmonella serotype typhi infections in the united states: Antimicrobial resistance on the rise. *JAMA* **283**, 2668–2673 (2000).
79. J, L. & J., J. Final report: Pilot of enhanced surveillance of enteric fever in England , Wales , and Northern Ireland. *London, UK Heal. Prot. Agency* (2007).
80. Crump, J. A., Luby, S. P. & Mintz, E. D. The global burden of typhoid fever. *Bull. World Health Organ.* **82**, 346–53 (2004).
81. Nga, T. V. T. *et al.* The decline of typhoid and the rise of non-typhoid salmonellae and fungal infections in a changing HIV landscape: bloodstream infection trends over 15 years in southern Vietnam. *Trans. R. Soc. Trop. Med. Hyg.* **106**, 26–34 (2012).
82. Reddy, E. A., Shaw, A. V & Crump, J. A. Community-acquired bloodstream infections in Africa: a systematic review and meta-analysis. *Lancet Infect. Dis.* **10**, 417–432 (2010).
83. Obaro, S. *et al.* Community Acquired Bacteremia in Young Children from Central Nigeria- A Pilot Study. *BMC Infect. Dis.* **11**, 137 (2011).
84. Thriemer, K. *et al.* The burden of invasive bacterial infections in Pemba, Zanzibar. *PLoS One* **7**, (2012).
85. Gasem, M. H., Dolmans, W. M., Keuter, M. M. & Djokomoeljanto, R. R. Poor food hygiene and housing as risk factors for typhoid fever in Semarang, Indonesia. *Trop. Med. Int. Health* **6**, 484–90 (2001).
86. Vollaard, A. M. *et al.* Risk factors for typhoid and paratyphoid fever in Jakarta, Indonesia. *JAMA* **291**, 2607–15 (2004).
87. Lin, F. Y. *et al.* The epidemiology of typhoid fever in the Dong Thap Province, Mekong Delta region of Vietnam. *Am. J. Trop. Med. Hyg.* **62**, 644–8 (2000).
88. Karkey, A. *et al.* The burden and characteristics of patients with Salmonella Typhi and Salmonella Paratyphi A presenting to a healthcare facility in a densely populated area of the Kathmandu valley. *Clin. Res.*
89. Wijedoru, L. P. M. *et al.* Typhoid Fever among Hospitalized Febrile Children in Siem Reap, Cambodia. *J. Trop. Pediatr.* **58**, 68–70 (2012).

90. Muhsen, K., Pasetti, M. F., Reymann, M. K., Graham, D. Y. & Levine, M. M. *Helicobacter pylori* infection affects immune responses following vaccination of typhoid-naïve us adults with attenuated salmonella typhi oral vaccine CVD 908-htrA. *J. Infect. Dis.* **209**, 1452–1458 (2014).
91. Dunstan, S. J. *et al.* Variation at HLA-DRB1 is associated with resistance to enteric fever. *Nat Genet* **46**, 1333–1336 (2014).
92. Nguyen, T. H. *et al.* Toll-like receptor 4 (TLR4) and typhoid fever in Vietnam. *PLoS One* **4**, 1–8 (2009).
93. Dunstan, S. J. *et al.* Host Susceptibility and Clinical Outcomes in Toll-Like Receptor 5 : Deficient Patients with Typhoid Fever in Vietnam. *J. Infect. Dis.* **191**, 1068–1071 (2005).
94. Alvarez, M. I. *et al.* Correction for Alvarez *et al.*, Human genetic variation in *VAC14* regulates *Salmonella* invasion and typhoid fever through modulation of cholesterol. *Proc. Natl. Acad. Sci.* **114**, E10849–E10850 (2017).
95. Pier, G. B. *et al.* Salmonella typhi uses CFTR to enter intestinal epithelial cells. *Nature* **393**, 79–82 (1998).
96. van de Vosse, E. *et al.* Susceptibility to typhoid fever is associated with a polymorphism in the cystic fibrosis transmembrane conductance regulator (CFTR). *Hum. Genet.* **118**, 138–40 (2005).
97. Sabbagh, S. C., Forest, C. G., Lepage, C., Leclerc, J.-M. & Daigle, F. So similar, yet so different: uncovering distinctive features in the genomes of Salmonella enterica serovars Typhimurium and Typhi. *FEMS Microbiol. Lett.* **305**, 1–13 (2010).
98. Parkhill, J. *et al.* Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. *October* **413**, (2001).
99. de Jong, H. K., Parry, C. M., van der Poll, T. & Wiersinga, W. J. Host-pathogen interaction in invasive Salmonellosis. *PLoS Pathog.* **8**, 1–9 (2012).
100. Valenzuela, L. M. *et al.* Pseudogenization of *sopA* and *sopE2* is functionally linked and contributes to virulence of Salmonella enterica serovar Typhi. *Infect. Genet. Evol.* **33**, 131–142 (2015).
101. Ortega, A. P. *et al.* Lose to win: *marT* pseudogenization in Salmonella enterica serovar Typhi contributed to the *surV*-dependent survival to H<sub>2</sub>O<sub>2</sub>, and inside human macrophage-like

- cells. *Infect. Genet. Evol.* **45**, 111–121 (2016).
102. Boyd, E. F., Porwollik, S., Blackmer, F., McClelland, M. & Icrobiol, J. C. L. I. N. M. Differences in Gene Content among Salmonella enterica Serovar Typhi Isolates. *Society* **41**, 3823–3828 (2003).
  103. Wain, J. *et al.* Molecular analysis of incHI1 antimicrobial resistance plasmids from Salmonella serovar Typhi strains associated with typhoid fever. *Antimicrob. Agents Chemother.* **47**, 2732–9 (2003).
  104. Phan, M.-D. *et al.* Variation in Salmonella enterica serovar typhi IncHI1 plasmids during the global spread of resistant typhoid fever. *Antimicrob. Agents Chemother.* **53**, 716–27 (2009).
  105. Bhutta, Z. A. Impact of age and drug resistance on mortality in typhoid fever. **76**, 214–217 (1996).
  106. Wu, S. yan *et al.* Salmonella spv locus suppresses host innate immune responses to bacterial infection. *Fish Shellfish Immunol.* **58**, 387–396 (2016).
  107. Guiney, D. G. & Fierer, J. The role of the spv genes in Salmonella pathogenesis. *Front. Microbiol.* **2**, 1–10 (2011).
  108. Silva, C., Puente, J. L. & Calva, E. Salmonella virulence plasmid: Pathogenesis and ecology. *Pathog. Dis.* **75**, 1–5 (2017).
  109. He, P. & Wu, S. Salmonella enterica serovar Typhi plasmid pRST98 enhances intracellular bacterial growth and S . typhi-induced macrophage cell death by suppressing autophagy. *Brazilian J. nfectious Dis.* **16**, 262–266 (2012).
  110. Wei, L., Wu, S., Li, Y., Chu, Y. & Huang, R. Salmonella enterica serovar Typhi plasmid impairs dendritic cell responses to infection. *Curr. Microbiol.* **65**, 133–140 (2012).
  111. Chu, Y. *et al.* A Salmonella enterica conjugative plasmid impairs autophagic flux in infected macrophages. *Microbes Infect.* **16**, 553–561 (2014).
  112. Baker, S. *et al.* Novel Linear Plasmid Mediates Flagellar Variation in Salmonella Typhi. *PLoS Pathog.* **3**, (2007).
  113. Galán, J. E. Interaction of Salmonella with host cells through the centisome 63 type III secretion system. *Curr. Opin. Microbiol.* **2**, 46–50 (1999).
  114. Johnson, R., Ravenhall, M., Pickard, D., Dougan, G. & Byrne, A. Comparison of Salmonella enterica Serovars Typhi and Typhimurium Reveals Typhoidal Serovar-Specific Responses.

- Infect. Immun.* **86**, 1–16 (2017).
115. Hensel, M. *et al.* Genes encoding putative effector proteins of the type III secretion system of *Salmonella* pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. *Mol. Microbiol.* **30**, 163–174 (1998).
  116. Forest, C. G., Ferraro, E., Sabbagh, S. C. & Daigle, F. Intracellular survival of *Salmonella enterica* serovar Typhi in human macrophages is independent of *Salmonella* pathogenicity island (SPI)-2. *Microbiology* **156**, 3689–98 (2010).
  117. Salazar-Gonzalez, R. M. & McSorley, S. J. *Salmonella* flagellin, a microbial target of the innate and adaptive immune system. *Immunol. Lett.* **101**, 117–122 (2005).
  118. Hu, X. *et al.* Vi capsular polysaccharide: Synthesis, virulence, and application. *Crit. Rev. Microbiol.* **43**, 440–452 (2017).
  119. Winter, S. E. *et al.* The flagellar regulator TviA reduces pyroptosis by *Salmonella enterica* serovar Typhi. *Infect. Immun.* **83**, 1546–1555 (2015).
  120. Gonzalez-Escobedo, G., Marshall, J. M. & Gunn, J. S. Chronic and acute infection of the gall bladder by *Salmonella* Typhi: understanding the carrier state. *Nat. Rev.* **9**, (2011).
  121. Proudfoot, A. Role of fimbriae-mediated adherence for neutrophil migration across *Escherichia coli* -infected epithelial cell layers. *Mol. Microbiol.* **30**, 725–735 (1998).
  122. Berrocal, L. *et al.* stg fimbrial operon from *S. Typhi* STH2370 contributes to association and cell disruption of epithelial and macrophage-like cells. *Biol. Res.* **48**, 1–8 (2015).
  123. Gonzales, A. M., Wilde, S. & Roland, K. L. Insights into the Roles of Long Polar Fimbriae and Stg Fimbriae in *Salmonella* Interactions with Enterocytes. *Infect. Immun.* **85**, 1–12 (2017).
  124. Saha, M. R., Ramamurthy, T., Dutta, P. & Mitra, U. Emergence of *Salmonella typhi* Vi antigen-negative strains in an epidemic of multidrug-resistant typhoid fever cases in Calcutta, India. *The National medical journal of India* **13**, 164 (2000).
  125. Baker, S. *et al.* Detection of Vi-Negative *Salmonella enterica* Serovar Typhi in the Peripheral Blood of Patients with Typhoid Fever in the Faisalabad Region of Pakistan. *Society* **43**, 4418–4425 (2005).
  126. Wain, J. *et al.* Vi Antigen Expression in *Salmonella enterica* Serovar Typhi Clinical Isolates from Pakistan. *J. Clin. Microbiol.* **43**, 1158–1165 (2005).

127. Miller, J. F., Mekalanos, J. J. & Falkow, S. Coordinate regulation and sensory transduction in the control of bacterial virulence. *Science* **243**, 916–922 (1989).
128. Pickard, D. *et al.* Characterization of defined ompR mutants of *Salmonella typhi*: ompR is involved in the regulation of Vi polysaccharide expression. *Infect. Immun.* **62**, 3984–93 (1994).
129. Arricau, N. *et al.* The RcsB-RcsC regulatory system of *Salmonella typhi* differentially modulates the expression of invasion proteins, flagellin and Vi antigen in response to osmolarity. *Mol. Microbiol.* **29**, 835–850 (1998).
130. Winter, S. E. *et al.* The TviA auxiliary protein renders the *Salmonella enterica* serotype Typhi RcsB regulon responsive to changes in osmolarity. *Mol. Microbiol.* **74**, 175–193 (2009).
131. Hirose, K. *et al.* Survival of Vi-capsulated and Vi-deleted. *FEMS Microbiol. Lett.* **147**, 259–265 (1997).
132. Hornick, R. B. *et al.* Typhoid fever: pathogenesis and immunologic control. *N. Engl. J. Med.* **283**, 686–91 (1970).
133. Hart, P. J. *et al.* Differential Killing of *Salmonella enterica* Serovar Typhi by Antibodies Targeting Vi and Lipopolysaccharide O : 9 Antigen. *PLoS One* **11**, 1–17 (2016).
134. Thiem, V. D. *et al.* The Vi conjugate typhoid vaccine is safe, elicits protective levels of IgG anti-Vi, and is compatible with routine infant vaccines. *Clin. Vaccine Immunol.* **18**, 730–735 (2011).
135. Lin, F. Y. C. *et al.* The efficacy of a *Salmonella Typhi* Vi conjugate vaccine in two-to-five-year-old children. *N. Engl. J. Med.* **344**, 1263–1269 (2011).
136. Song, J., Gao, X. & Galán, J. E. Structure and function of the *Salmonella Typhi* chimaeric A(2)B(5) typhoid toxin. *Nature* **499**, 350–4 (2013).
137. Song, J., Gao, X. & Galán, J. E. Structure and function of the *Salmonella Typhi* chimaeric A(2)B(5) typhoid toxin. *Nature* **499**, 350–4 (2013).
138. Haghjoo, E. & Galán, J. E. *Salmonella typhi* encodes a functional cytolethal distending toxin that is delivered into host cells by a bacterial-internalization pathway. *Proc. Natl. Acad. Sci. U. S. A.* **101**, 4614–9 (2004).
139. Galán, J. E. Typhoid toxin provides a window into typhoid fever and the biology of *Salmonella Typhi*. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 6338–44 (2016).

140. Jombart, T. *et al.* Characterization of anti-Salmonella enterica serotype Typhi antibody responses in bacteremic Bangladeshi patients by an immunoaffinity proteomics-based technology. *Infect. Immun.* **5**, 2894–2901 (2014).
141. Del, L. *et al.* The Typhoid Toxin Promotes Host Survival and the Establishment of a Persistent Asymptomatic Infection. 1–25 (2016). doi:10.1371/journal.ppat.1005528
142. Roy, D., Steyer, G. J., Gargsha, M., Stone, M. E. & Wilson, L. Host adaptation of a bacterial toxin from the human pathogen Salmonella Typhi. *Cell* **159**, 1290–1299 (2014).
143. Chong, A., Lee, S., Yang, Y. A. & Song, J. The role of typhoid toxin in Salmonella Typhi virulence. *Yale J. Biol. Med.* **90**, 283–290 (2017).
144. Song, J. *et al.* A mouse model for the human pathogen salmonella typhi. *Cell Host Microbe* **8**, 369–376 (2010).
145. Hacker, J. *et al.* Ecological fitness, genomic islands and bacterial pathogenicity A Darwinian view of the evolution of microbes. *EMBO Rep.* **2**, 376–381 (2001).
146. Feng, Y., Chien, K. Y., Chen, H. L. & Chiu, C. H. Pseudogene recoding revealed from proteomic analysis of salmonella serovars. *J. Proteome Res.* **11**, 1715–1719 (2012).
147. Maurelli, A. T. Black holes, antivirulence genes, and gene inactivation in the evolution of bacterial pathogens. *FEMS Microbiol. Lett.* **267**, 1–8 (2007).
148. Baker, S. & Dougan, G. The genome of Salmonella enterica serovar Typhi. *Clin. Infect. Dis.* **45 Suppl 1**, S29-33 (2007).
149. Freeman, J. a, Ohl, M. E. & Miller, S. I. The Salmonella enterica Serovar Typhimurium Translocated Effectors SseJ and SifB Are Targeted to the Salmonella The Salmonella enterica Serovar Typhimurium Translocated Effectors SseJ and SifB Are Targeted to the Salmonella -Containing Vacuole. *Infect. Immun.* **71**, 418–427 (2003).
150. Ohlson, M. B. *et al.* SseJ Deacylase Activity by Salmonella enterica Serovar Typhimurium Promotes Virulence in Mice. *Infect. Immun.* **73**, 6249–6259 (2005).
151. Eswarappa, S. M. *et al.* Differentially Evolved Genes of Salmonella Pathogenicity Islands : Insights into the Mechanism of Host Specificity in Salmonella. *PLoS One* **3**, e3829 (2008).
152. Trombert, A. N., Berrocal, L., Fuentes, J. A. & Mora, G. C. S. Typhimurium sseJ gene decreases the S. Typhi cytotoxicity toward cultured epithelial cells. *BMC Microbiol.* **10**, 312 (2010).



153. Trombert, A. N., Rodas, P. I. & Mora, G. C. Reduced invasion to human epithelial cell lines of Salmonella enterica serovar Typhi carrying S. Typhimurium sopD2. *FEMS Microbiol. Lett.* **322**, 150–156 (2011).
154. Johnson, R. *et al.* The Type III Secretion System Effector SptP of Salmonella enterica Serovar Typhi. **199**, 1–18 (2017).
155. Libby, S. J. *et al.* Humanized nonobese diabetic-scid IL2rgammanull mice are susceptible to lethal Salmonella Typhi infection. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 15589–94 (2010).
156. Mian, M. F., Pek, E. A., Chenoweth, M. J., Coombes, B. K. & Ashkar, A. A. Humanized mice for Salmonella typhi infection: new tools for an old problem. *Virulence* **2**, 248–52 (2011).
157. Brehm, M. A., Shultz, L. D., Luban, J. & Greiner, D. L. Overcoming current limitations in humanized mouse research. *J. Infect. Dis.* **208 Suppl**, S125-30 (2013).
158. Mathur, R. *et al.* A mouse model of salmonella typhi infection. *Cell* **151**, 590–602 (2012).
159. Levine, M. M., Tacket, C. O. & Sztein, M. B. Host-Salmonella interaction: human trials. *Microbes Infect.* **3**, 1271–1279 (2001).
160. Woodward, W. E. Volunteer studies of typhoid fever and vaccines. *Trans. R. Soc. Trop. Med. Hyg.* **74**, 553–556 (1980).
161. Hornick, R. B. *et al.* Typhoid fever: pathogenesis and immunologic control. 2. *N. Engl. J. Med.* **283**, 739–46 (1970).
162. Salerno-Goncalves, R. *et al.* Challenge of humans with wild-type Salmonella enterica serovar typhi elicits changes in the activation and homing characteristics of mucosal-associated invariant T cells. *Front. Immunol.* **8**, 1–13 (2017).
163. Fresnay, S. *et al.* Salmonella Typhi-specific multifunctional CD8<sup>+</sup> T cells play a dominant role in protection from typhoid fever in humans. *J. Transl. Med.* **14**, 1–14 (2016).
164. Darton, T. C. *et al.* Rapidly Escalating Hecpudin and Associated Serum Iron Starvation Are Features of the Acute Response to Typhoid Infection in Humans. *PLoS Negl. Trop. Dis.* **9**, e0004029 (2015).
165. Fresnay, S. *et al.* Importance of Salmonella typhi-responsive CD8<sup>+</sup> T cell immunity in a human typhoid fever challenge model. *Front. Immunol.* **8**, (2017).
166. Juel, H. B. *et al.* Salmonella Typhi bactericidal antibodies reduce disease severity but do not

- protect against typhoid fever in a controlled human infection model. *Front. Immunol.* **8**, 1–11 (2018).
167. McArthur, M. A. *et al.* Activation of Salmonella Typhi-Specific Regulatory T Cells in Typhoid Disease in a Wild-Type S. Typhi Challenge Model. *PLoS Pathog.* **11**, 1–18 (2015).
  168. Darton, T. C. *et al.* Using a Human Challenge Model of Infection to Measure Vaccine Efficacy: A Randomised, Controlled Trial Comparing the Typhoid Vaccines M01ZH09 with Placebo and Ty21a. *PLoS Negl. Trop. Dis.* **10**, 1–27 (2016).
  169. Toapanta, F. R. *et al.* Oral Wild-Type Salmonella Typhi Challenge Induces Activation of Circulating Monocytes and Dendritic Cells in Individuals Who Develop Typhoid Disease. *PLoS Negl. Trop. Dis.* **9**, e0003837 (2015).
  170. Parry, C., Hien, T., Dougan, G., White, N. & Farrar, J. Typhoid fever. *N. Engl. J. Med.* **347**, 1770–1782 (2002).
  171. Tiremu, S. N., Revankar, S. A. & Pai, S. A. An Unusual Cause of Granulomatous Ileitis – With a Brief History of the Pathology of Typhoid. *Int. J. Surg. Pathol.* **26**, 240–241 (2018).
  172. Patel, S. & McCormick, B. A. Mucosal inflammatory response to Salmonella typhimurium infection. *Front. Immunol.* **5**, 1–10 (2014).
  173. Keestra-Gounder, A. M., Tsolis, R. M. & Bäumlér, A. J. Now you see me, now you don't: the interaction of Salmonella with innate immune receptors. *Nat. Rev. Microbiol.* **13**, 206–216 (2015).
  174. Wangdi, T., Winter, S. E. & Bäumlér, A. J. Typhoid fever 'you can't hit what you can't see'. *Gut Microbes* **3**, 88–92 (2012).
  175. Wilson, R. P. *et al.* The Vi capsular polysaccharide prevents complement receptor 3-mediated clearance of Salmonella enterica serotype Typhi. *Infect. Immun.* **79**, 830–837 (2011).
  176. Crawford, R. W. *et al.* Loss of very-long O-antigen chains optimizes capsule-mediated immune evasion by Salmonella enterica serovar Typhi. *MBio* **4**, 1–8 (2013).
  177. Ward, P. A. The dark side of C5a in sepsis. *Nat. Rev. Immunol.* **4**, 133–142 (2004).
  178. Raffatellu, M. *et al.* The Vi Capsular Antigen of Salmonella enterica Serotype Typhi Reduces Toll-Like Receptor-Dependent Interleukin-8 Expression in the Intestinal Mucosa. *Society* **73**, 3367–3374 (2005).
  179. Wilson, R. P. *et al.* The Vi-capsule prevents Toll-like receptor 4 recognition of Salmonella.

- Cell. Microbiol.* **10**, 876–90 (2008).
180. Mollnes, T. E. *et al.* Essential role of the C5a receptor in E coli – induced oxidative burst and phagocytosis revealed by a novel lepirudin-based human whole blood model of inflammation  
Essential role of the C5a receptor in E coli – induced oxidative burst and phagocytosis rev.  
*Blood* **100**, 1869–1877 (2013).
  181. Wangdi, T. *et al.* The Vi Capsular Polysaccharide Enables Salmonella enterica Serovar Typhi to Evade Microbe-Guided Neutrophil Chemotaxis. *PLoS Pathog.* **10**, (2014).
  182. Dougan, G. & Baker, S. Salmonella enterica serovar Typhi and the pathogenesis of typhoid fever. *Annu Rev Microbiol* **68**, 317–336 (2014).
  183. Winter, S. E., Raffatellu, M., Wilson, R. P., Rüssmann, H. & Bäumlér, A. J. The Salmonella enterica serotype Typhi regulator TviA reduces interleukin-8 production in intestinal epithelial cells by repressing flagellin secretion. *Cell. Microbiol.* **10**, 247–61 (2008).
  184. Atif, S. M., Winter, S. E., Winter, M. G., McSorley, S. J. & Bäumlér, A. J. Salmonella enterica serovar typhi impairs CD4 T cell responses by reducing antigen availability. *Infect. Immun.* **82**, 2247–2254 (2014).
  185. Knodler, L. A. *et al.* Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proc. Natl. Acad. Sci.* **107**, 17733–17738 (2010).
  186. Winter, S. E. *et al.* Salmonella enterica Serovar Typhi Conceals the Invasion-Associated Type Three Secretion System from the Innate Immune System by Gene Regulation. *PLoS Pathog.* **10**, (2014).
  187. Khan, A. Q. *et al.* Salmonella typhi rpoS mutant is less cytotoxic than the parent strain but survives inside resting THP-1 macrophages. *FEMS Microbiol. Lett.* **161**, 201–208 (1998).
  188. Hashizume, T. *et al.* Peyer’s Patches Are Required for Intestinal Immunoglobulin A Responses to Salmonella spp. *Infect. Immun.* **76**, 927–934 (2008).
  189. Kohbata, S., Yokoyama, H. & Yabuuchi, E. Cytopathogenic effect of Salmonella typhi GIFU 10007 on M cells of murine ileal Peyer’s patches in ligated ileal loops: an ultrastructural study. *Microbiol. Immunol.* **30**, 1225–1237 (1986).
  190. Martinoli, C., Chiavelli, A. & Rescigno, M. Entry Route of Salmonella typhimurium Directs the Type of Induced Immune Response. *Immunity* **27**, 975–984 (2007).
  191. Nickerson, K. P. *et al.* Salmonella Typhi Colonization Provokes Extensive Transcriptional

Changes Aimed at Evading Host Mucosal Immune Defense During Early Infection of Human Intestinal Tissue. *EBioMedicine* **31**, 92–109 (2018).

192. Sztein, M. B., Salerno-Goncalves, R. & McArthur, M. A. Complex adaptive immunity to enteric fevers in humans: Lessons learned and the path forward. *Front. Immunol.* **5**, (2014).
193. Boumart, Z., Velge, P. & Wiedemann, A. Multiple invasion mechanisms and different intracellular Behaviors: A new vision of Salmonella-host cell interaction. *FEMS Microbiol. Lett.* **361**, 1–7 (2014).
194. Ortiz, V., Isibasi, A., Garcia-Ortigoza, E. & Kumate, J. Immunoblot detection of class-specific humoral immune response to outer membrane proteins isolated from Salmonella typhi in humans with typhoid fever. *J. Clin. Microbiol.* **27**, 1640–1645 (1989).
195. Jesudason, M. V., Sridharan, G., Arulselvan, R., Babu, P. G. & John, T. J. Diagnosis of typhoid fever by the detection of anti-LPS & anti-flagellin antibodies by ELISA. *Indian J. Med. Res.* **107**, 204–207 (1998).
196. Tang, S., Abubakar, S., Devi, S., Puthucheary, S. & Pang, T. Induction and Characterization of Heat Shock Proteins of Salmonella typhi and Their Reactivity with Sera from Patients with Typhoid Fever. *Infect. Immun.* **65**, 2983–2986 (1997).
197. Herath, H. M. T. U. Early diagnosis of typhoid fever by the detection of salivary IgA. *J. Clin. Pathol.* **56**, 694 LP-698 (2003).
198. Levine, M. M. *et al.* Diagnostic value of the Widal test in areas endemic for typhoid fever. *Am. J. Trop. Med. Hyg.* **27**, 795–800 (1978).
199. Sheikh, A. *et al.* Salmonella enterica serovar typhi-specific immunoglobulin A antibody responses in plasma and antibody in lymphocyte supernatant specimens in Bangladeshi patients with suspected typhoid fever. *Clin. Vaccine Immunol.* **16**, 1587–1594 (2009).
200. Losonsky, G. A., Ferreccio, C., Kotloff, K. L., Kaintuck, S. & Robbins, J. B. Development and Evaluation of an Enzyme-Linked Immunosorbent Assay for Serum Vi Antibodies for Detection of Chronic Salmonella typhi Carriers. *Microbiology* **25**, 2266–2269 (1987).
201. Marmion, D. E., Naylor, G. R. & Stewart, I. O. Second attacks of typhoid fever. *J. Hyg. (Lond)*. **51**, 260–267 (1953).
202. Dupont, H. L. *et al.* Studies of immunity in typhoid fever. Protection induced by killed oral antigens or by primary infection. *Bull. World Health Organ.* **44**, 667–72 (1971).

203. Levine, M. M., Ferreccio, C., Black, R. E., Tacket, C. O. & Germanier, R. Progress in vaccines against typhoid fever. *Rev. Infect. Dis.* **11 Suppl 3**, S552-67 (1989).
204. Waddington, C. S. *et al.* An outpatient, ambulant-design, controlled human infection model using escalating doses of salmonella typhi challenge delivered in sodium bicarbonate solution. *Clin. Infect. Dis.* **58**, 1230–1240 (2014).
205. Ivanoff, B., Levine, M. M. & Lambert, P. H. Vaccination against typhoid fever: present status. *Bull. World Health Organ.* **72**, 957–971 (1994).
206. Szu, S. C., Klugman, K. P. & Hunt, S. Re-examination of immune response and estimation of anti-Vi IgG protective threshold against typhoid fever-based on the efficacy trial of Vi conjugate in young children. *Vaccine* **32**, 2359–2363 (2014).
207. Pulickal, A. S. *et al.* Kinetics of the natural, humoral immune response to Salmonella enterica serovar Typhi in Kathmandu, Nepal. *Clin. Vaccine Immunol.* **16**, 1413–9 (2009).
208. Salerno-Goncalves, R., Rezwan, T. & Sztein, M. B. B cells modulate mucosal associated invariant T cell immune responses. *Front. Immunol.* **4**, 511 (2014).
209. Wahid, R., Salerno-Goncalves, R., Tacket, C. O., Levine, M. M. & Sztein, M. B. Generation of specific effector and memory T cells with gut- and secondary lymphoid tissue- homing potential by oral attenuated CVD 909 typhoid vaccine in humans. *Mucosal Immunol.* **1**, 389–398 (2008).
210. Wahid, R., Salerno-Goncalves, R., Tacket, C. O., Levine, M. M. & Sztein, M. B. Cell-mediated immune responses in humans after immunization with one or two doses of oral live attenuated typhoid vaccine CVD 909. *Vaccine* **25**, 1416–1425 (2007).
211. Sztein, M. B., Tanner, M. K., Polotsky, Y., Orenstein, J. M. & Levine, M. M. Cytotoxic T lymphocytes after oral immunization with attenuated vaccine strains of Salmonella typhi in humans. *J. Immunol.* **155**, 3987–3993 (1995).
212. Salerno-Goncalves, R. *et al.* Concomitant induction of CD4+ and CD8+ T cell responses in volunteers immunized with Salmonella enterica serovar typhi strain CVD 908-htrA. *J. Immunol.* **170**, 2734–2741 (2003).
213. Salerno-Goncalves, R., Fernandez-Vina, M., Lewinsohn, D. M. & Sztein, M. B. Identification of a human HLA-E-restricted CD8+ T cell subset in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine. *J. Immunol.* **173**, 5852–5862 (2004).

214. Salerno-Goncalves, R., Pasetti, M. F. & Sztein, M. B. Characterization of CD8(+) effector T cell responses in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine. *J. Immunol.* **169**, 2196–2203 (2002).
215. Salerno-Goncalves, R., Wahid, R. & Sztein, M. B. Immunization of volunteers with Salmonella enterica serovar Typhi strain Ty21a elicits the oligoclonal expansion of CD8+ T cells with predominant Vbeta repertoires. *Infect. Immun.* **73**, 3521–3530 (2005).
216. Salerno-Goncalves, R., Wahid, R. & Sztein, M. B. Ex Vivo kinetics of early and long-term multifunctional human leukocyte antigen E-specific CD8+ cells in volunteers immunized with the Ty21a typhoid vaccine. *Clin. Vaccine Immunol.* **17**, 1305–1314 (2010).
217. Tacket, C. O. *et al.* Safety of live oral Salmonella typhi vaccine strains with deletions in htrA and aroC aroD and immune response in humans. *Infect. Immun.* **65**, 452–6 (1997).
218. Wahid, R., Simon, R., Zafar, S. J., Levine, M. M. & Sztein, M. B. Live oral typhoid vaccine Ty21a induces cross-reactive humoral immune responses against Salmonella enterica serovar paratyphi A and S. paratyphi B in humans. *Clin. Vaccine Immunol.* **19**, 825–834 (2012).
219. Tacket, C. O., Pasetti, M. F., Sztein, M. B., Livio, S. & Levine, M. M. Immune Responses to an Oral Typhoid Vaccine Strain That Is Modified to Constitutively Express Vi Capsular Polysaccharide. *J. Infect. Dis.* **190**, 565–570 (2004).
220. Kirkpatrick, B. D. *et al.* Comparison of the Antibodies in Lymphocyte Supernatant and Antibody-Secreting Cell Assays for Measuring Intestinal Mucosal Immune Response to a Novel Oral Typhoid Vaccine (M01ZH09). *Clin. Vaccine Immunol.* **12**, 1127–1129 (2005).
221. Tacket, C. O. *et al.* Phase 2 clinical trial of attenuated Salmonella enterica serovar typhi oral live vector vaccine CVD 908-htrA in U.S. volunteers. *Infect. Immun.* **68**, 1196–201 (2000).
222. Kantele, A., Pakkanen, S. H., Karttunen, R. & Kantele, J. M. Head-to-head comparison of humoral immune responses to Vi capsular polysaccharide and Salmonella Typhi Ty21a typhoid vaccines-a randomized trial. *PLoS One* **8**, e60583 (2013).
223. Penttila, I. A., Gibson, C. E., Leong, A. Y., Zola, H. & LaBrooy, J. T. Cell surface determinants on typhoid-specific B cells isolated from peripheral blood after oral Ty21a typhoid vaccination in humans. *Immunol. Cell Biol.* **73**, 198–204 (1995).
224. Kantele, A. Peripheral blood antibody-secreting cells in the evaluation of the immune response to an oral vaccine. *J. Biotechnol.* **44**, 217–224 (1996).

225. Kantele, A. Antibody-secreting cells in the evaluation of the immunogenicity of an oral vaccine. *Vaccine* **8**, 321–326 (1990).
226. Gordon, M. A. Salmonella infections in immunocompromised adults. *J. Infect.* **56**, 413–22 (2008).
227. Lammas, D. A., Casanova, J. L. & Kumararatne, D. S. Clinical consequences of defects in the IL-12-dependent interferon-gamma (IFN-gamma) pathway. *Clin. Exp. Immunol.* **121**, 417–425 (2000).
228. de Jong, R. *et al.* Severe mycobacterial and Salmonella infections in interleukin-12 receptor-deficient patients. *Science* **280**, 1435–1438 (1998).
229. Dunstan, S. J. *et al.* Genes of the Class II and Class III Major Histocompatibility Complex Are Associated with Typhoid Fever in Vietnam. *J. Infect. Dis.* **183**, 261–268 (2001).
230. Butler, T., Ho, M., Acharya, G., Tiwari, M. & Gallati, H. Interleukin-6, gamma interferon, and tumor necrosis factor receptors in typhoid fever related to outcome of antimicrobial therapy. *Antimicrob. Agents Chemother.* **37**, 2418–2421 (1993).
231. Keuter, M. *et al.* Patterns of Proinflammatory Cytokines and Inhibitors during Typhoid Fever. *J. Infect. Dis.* **169**, 1306–1311 (1994).
232. Gal-Mor, O. *et al.* Molecular and cellular characterization of a Salmonella enterica serovar paratyphi A outbreak strain and the human immune response to infection. *Clin. Vaccine Immunol.* **19**, 146–156 (2012).
233. Kirkpatrick, B. D. *et al.* The Novel Oral Typhoid Vaccine M01ZH09 in 2 Vaccine Presentations Is Well Tolerated and Highly Immunogenic in 2 Vaccine Presentation. *J. Infect. Dis.* **192**, 360–366 (2005).
234. Wyant, T. L., Tanner, M. K. & Sztein, M. B. Flagella Are Potent Inducers of Proinflammatory Cytokine Secretion by Human Monocytes. *Infect. Immun.* **67**, 3619 LP-3624 (1999).
235. Viret, J. F. *et al.* Mucosal and systemic immune responses in humans after primary and booster immunizations with orally administered invasive and noninvasive live attenuated bacteria. *Infect. Immun.* **67**, 3680–3685 (1999).
236. Sztein, M. B. Cell-mediated immunity and antibody responses elicited by attenuated Salmonella enterica Serovar Typhi strains used as live oral vaccines in humans. *Clin. Infect.*

*Dis.* **45 Suppl 1**, S15-9 (2007).

237. Dham, S. K. & Thompson, R. A. Studies of cellular and humoral immunity in typhoid fever and TAB vaccinated subjects. *Clin. Exp. Immunol.* **48**, 389–395 (1982).
238. Spanò, S. Host restriction in Salmonella: Insights from Rab GTPases. *Cell. Microbiol.* **16**, 1321–1328 (2014).
239. Spanò, S., Gao, X., Hannemann, S. & Haven, N. A Bacterial Pathogen Targets a Host Rab-Family GTPase Defense Pathway with a GAP. *Cell Host Microbe* **19**, 216–226 (2016).
240. Pfeffer, S. R. Rab GTPase regulation of membrane identity. *Curr. Opin. Cell Biol.* **25**, 414–419 (2013).
241. Barr, F. A. Rab GTPases and membrane identity: Causal or inconsequential? *J. Cell Biol.* **202**, 191–199 (2013).
242. Spanò, S. & Galán, J. E. A Rab32-dependent pathway contributes to Salmonella typhi host restriction. *Science* **338**, 960–3 (2012).
243. Spanò, S., Liu, X. & Galán, J. E. Proteolytic targeting of Rab29 by an effector protein distinguishes the intracellular compartments of human-adapted and broad-host Salmonella. *PNAS* **108**, 18418–18423 (2011).
244. Schwan, W. R., Huang, X., Hu, L. & Dennis, J. Differential Bacterial Survival , Replication , and Apoptosis-Inducing Ability of Salmonella Serovars within Human and Murine Macrophages. *Infect. Immun.* **68**, 1005–1013 (2000).
245. EDSALL, G. *et al.* Studies on infection and immunity in experimental typhoid fever. I. Typhoid fever in chimpanzees orally infected with Salmonella typhosa. *J. Exp. Med.* **112**, 143–166 (1960).
246. Chang, S. J., Song, J. & Galán, J. E. Receptor-Mediated Sorting of Typhoid Toxin during Its Export from Salmonella Typhi-Infected Cells. *Cell Host Microbe* **20**, 682–689 (2016).
247. Deng, L. *et al.* Host adaptation of a bacterial toxin from the human pathogen salmonella typhi. *Cell* **159**, 1290–1299 (2014).
248. Brooks, J. The sad and tragic life of Typhoid Mary. *Can. Med. Assoc. J.* **154**, 915–916 (1996).
249. Crump, J. A., Mintz, E. D., Crump, J. A. & Mintz, E. D. Global Trends in Typhoid and Paratyphoid Fever. *Clin. Infect. Dis.* **50**, 241–246 (2010).



250. Gunn, J. S. *et al.* Salmonella chronic carriage: epidemiology, diagnosis, and gallbladder persistence. *Trends Microbiol.* **22**, 648–55 (2014).
251. Crawford, R. W. *et al.* Gallstones play a significant role in Salmonella spp. gallbladder colonization and carriage. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 4353–8 (2010).
252. Crawford, R. W., Gibson, D. L., Kay, W. W. & Gunn, J. S. Identification of a bile-induced exopolysaccharide required for Salmonella biofilm formation on gallstone surfaces. *Infect. Immun.* **76**, 5341–9 (2008).
253. Gonzalez-escobedo, G. & Gunn, S. Gallbladder Epithelium as a Niche for Chronic Salmonella Carriage. *Infect. Immun.* **81**, 2920–2930 (2013).
254. Wernberg, J. A. & Lucarelli, D. D. Gallbladder cancer. *Surg. Clin. North Am.* **94**, 343–360 (2014).
255. Nagaraja, V. & Eslick, G. D. Alimentary Pharmacology and Therapeutics Systematic review with meta-analysis: the relationship between chronic Salmonella typhi carrier status and gallbladder cancer. *Aliment. Pharmacol. Ther.* **39**, 745–750 (2014).
256. Di Domenico, E. G., Cavallo, I., Pontone, M., Toma, L. & Ensoli, F. Biofilm producing Salmonella typhi: Chronic colonization and development of gallbladder cancer. *Int. J. Mol. Sci.* **18**, (2017).
257. Nstrom, E. *et al.* Diagnostic metabolite biomarkers of chronic typhoid carriage. *PLoS Negl. Trop. Dis.* **12**, 1–15 (2018).
258. Bokkenheuser, V. Detection of Typhoid Carriers. *Am. J. Public Heal. Nations Heal.* **54**, 477–486 (1964).
259. Lanata, C. F. *et al.* Vi serology in detection of chronic Salmonella typhi carriers in an endemic area. *Lancet (London, England)* **2**, 441–3 (1983).
260. Costerton, A. J. W., Stewart, P. S. & Greenberg, E. P. Bacterial Biofilms: A Common Cause of Persistent Infections. *Science (80-. )*. **284**, 1318–1322 (2011).
261. Main, R. G. Treatment of the Chronic Alimentary Enteric Carrier. *Br. Med. J.* **1**, 328–333 (1961).
262. Ristori, C. *et al.* Persistence of the Salmonella typhi-paratyphi carrier state after gallbladder removal. *Bull. Pan Am. Health Organ.* **16**, 361–366 (1982).
263. Nath, G., Pratap, C. B., Patel, S. K., Gulati, A. K. & Tripathi, S. K. Isolation of Salmonella

- typhi from apparently healthy liver. *Infect. Genet. Evol.* **11**, 2103–2105 (2011).
264. Parry, C. M., Wijedoru, L., Arjyal, A. & Baker, S. The utility of diagnostic tests for enteric fever in endemic locations. *Expert Rev. Anti. Infect. Ther.* **9**, 711–725 (2011).
  265. Baker, S., Favorov, M. & Dougan, G. Searching For the Elusive Typhoid Diagnostic. *BMC Infect. Dis.* **10**, 1–23 (2010).
  266. Khanam, F. *et al.* Evaluation of a typhoid/paratyphoid diagnostic assay (TPTest) detecting anti-Salmonella IgA in secretions of peripheral blood lymphocytes in patients in Dhaka, Bangladesh. *PLoS Negl. Trop. Dis.* **7**, e2316 (2013).
  267. Islam, K. *et al.* Comparison of the Performance of the TPTest, Tubex, Typhidot and Widal Immunodiagnostic Assays and Blood Cultures in Detecting Patients with Typhoid Fever in Bangladesh, Including Using a Bayesian Latent Class Modeling Approach. *PLoS Negl. Trop. Dis.* **10**, e0004558 (2016).
  268. Wain, J. & Hosoglu, S. The laboratory diagnosis of enteric fever. *J. Infect. Dev. Ctries.* **2**, 421–425 (2008).
  269. Guerra-Caceres, J. G., Gotuzzo-Herencia, E., Crosby-Dagnino, E., Miro-Quesada, M. & Carrillo-Parodi, C. Diagnostic value of bone marrow culture in typhoid fever. *Trans. R. Soc. Trop. Med. Hyg.* **73**, 680–683 (1979).
  270. Antillon, M., Saad, N. J., Baker, S., Pollard, A. J. & Pitzer, V. E. The Relationship Between Blood Sample Volume and Diagnostic Sensitivity of Blood Culture for Typhoid and Paratyphoid Fever : A Systematic Review and. *J. Infect. Dis.* **218**, 255–267 (2018).
  271. Mogasale, V., Ramani, E., Mogasale, V. V & Park, J. What proportion of Salmonella Typhi cases are detected by blood culture ? A systematic literature review. *Ann. Clin. Microbiol. Antimicrob.* **15**, 1–8 (2016).
  272. Wain, J. *et al.* Quantitation of bacteria in bone marrow from patients with typhoid fever: relationship between counts and clinical features. *J. Clin. Microbiol.* **39**, 1571–1576 (2001).
  273. Brooks, A. W. *et al.* Bacteremic typhoid fever in children in an Urban slum, Bangladesh. *Emerg. Infect. Dis.* **11**, 326–329 (2005).
  274. Cho, H., Park, M., Na, D. & Moon, H. Detection of Salmonella typhi in the Blood of Patients with Typhoid Fever by Polymerase Chain Reaction. *J. Clin. Microbiol.* **31**, 1439–1443 (1993).

275. Massi, M. N. *et al.* Quantitative detection of *Salmonella enterica* serovar Typhi from blood of suspected typhoid fever patients by real-time PCR. *Int. J. Med. Microbiol.* **295**, 117–120 (2005).
276. Massi, M. N. *et al.* Rapid diagnosis of typhoid fever by PCR assay using one pair of primers from flagellin gene of *Salmonella typhi*. *J. Infect. Chemother. Off. J. Japan Soc. Chemother.* **9**, 233–237 (2003).
277. Haque, A., Ahmed, J. & Qureshi, J. A. Early detection of typhoid by polymerase chain reaction. *Ann. Saudi Med.* **19**, 337–340 (1999).
278. Chaudhry, R., Laxmi, B. V., Nisar, N., Ray, K. & Kumar, D. Standardisation of polymerase chain reaction for the detection of *Salmonella typhi* in typhoid fever. *J. Clin. Pathol.* **50**, 437–439 (1997).
279. Ambati, S. R., Nath, G. & Das, B. K. Diagnosis of Typhoid Fever by Polymerase Chain Reaction. *Blood* **74**, 909–913 (2007).
280. Hatta, M. & Smits, H. L. Detection of *Salmonella Typhi* by Nested Polymerase Chain Reaction in Blood, Urine, and Stool Samples. *Trop. Med.* **76**, 139–143 (2007).
281. Nga, T. V. T. *et al.* The sensitivity of real-time PCR amplification targeting invasive *Salmonella* serovars in biological specimens. *BMC Infect. Dis.* **10**, 125 (2010).
282. Nizami, S. Q., Bhutta, Z. A., Siddiqui, A. A. & Lubbad, L. Enhanced detection rate of typhoid fever in children in a periurban slum in Karachi, Pakistan using polymerase chain reaction technology. *Scand. J. Clin. Lab. Invest.* **66**, 429–436 (2006).
283. Zhou, L. & Pollard, A. J. A novel method of selective removal of human DNA improves PCR sensitivity for detection of *Salmonella Typhi* in blood samples. *BMC Infect. Dis.* **12**, 1 (2012).
284. Zhou, L. *et al.* Development and Evaluation of a Blood Culture PCR Assay for Rapid Detection of *Salmonella Paratyphi A* in Clinical Samples. *PLoS One* **11**, e0150576 (2016).
285. Olopoenia, L. A. & King, A. L. Widal agglutination test - 100 years later: still plagued by controversy. *Postgrad. Med. J.* **76**, 80–84 (2000).
286. Wijedoru, L., Mallett, S. & Cm, P. Rapid diagnostic tests for typhoid and paratyphoid ( enteric ) fever. *Cochrane database Syst. Rev.* **5**, (2017).
287. Kiratisin, P. Bacteraemia due to non-typhoidal *Salmonella* in Thailand: clinical and microbiological analysis. *Trans. R. Soc. Trop. Med. Hyg.* **102**, 384–388 (2008).

288. Gröschel, D. H. M. & Hornick, R. B. Who Introduced Typhoid Vaccination : Almroth Wright or Richard Pfeiffer? *Rev. Infect. Dis.* **3**, 1251–1254 (1981).
289. Levine, M. M. Progress in Vaccines against Typhoid Fever. **11**, (1989).
290. Levine, M. M. Typhoid Vaccines Ready for Implementation : A History. 403–405 (2009).
291. Germanier, R. & Fürer, E. Characteristics of the attenuated oral vaccine strain ‘S. typhi’ Ty 21a. *Dev. Biol. Stand.* **53**, 3–7 (1983).
292. Germanier, R. & Fürer, E. Isolation and Characterization of Gal E Mutant Ty 21a of *Salmonella typhi*: A Candidate Strain for a Live, Oral Typhoid Vaccine. *J. Infect. Dis.* **131**, 553–558 (1975).
293. Coynault, C., Robbe-Saule, V. & Norel, F. Virulence and vaccine potential of *Salmonella typhimurium* mutants deficient in the expression of the RpoS (sigma S) regulon. *Mol. Microbiol.* **22**, 149–160 (1996).
294. Gilman, R. H., Hornick, R. B. & Woodward, W. E. Evaluation of a UDP-Glucose-4-Epimeraseless Mutant of *Salmonella typhi* as a Live Oral Vaccine. **136**, 717–723 (1977).
295. Guzman, C. A. *et al.* Vaccines against typhoid fever. *Vaccine* **24**, 3804–3811 (2006).
296. Levine, M. M. *et al.* Duration of efficacy of Ty21a, attenuated *Salmonella typhi* live oral vaccine. *Vaccine* **17**, 22–27 (1999).
297. Ferreccio, C., Levine, M. M., Rodriguez, H. & Contreras, R. Comparative Efficacy of Two, Three, or Four Doses of TY21a Live Oral Typhoid Vaccine in Enteric-Coated Capsules: A Field Trial in an Endemic Area. *J. Infect. Dis.* **159**, 766–769 (1989).
298. Wyant, T. L., Tanner, M. K. & Sztein, M. B. *Salmonella typhi* flagella are potent inducers of proinflammatory cytokine secretion by human monocytes. *Infect. Immun.* **67**, 3619–24 (1999).
299. Levine, M. M. ., Ferreccio, C., Black, R. E., Tacket, C. & Germanier, R. Progress in Vaccines against Typhoid Fever. *Rev. Infect. Dis.* **11**, S552–S567 (1989).
300. Forrest, B. D. Indirect measurement of intestinal immune responses to an orally administered attenuated bacterial vaccine. *Infect. Immun.* **60**, 2023–2029 (1992).
301. Lundgren, A., Kaim, J. & Jertborn, M. Parallel analysis of mucosally derived B- and T-cell responses to an oral typhoid vaccine using simplified methods. *Vaccine* **27**, 4529–4536 (2009).

302. Lundin, B. S., Johansson, C. & Svennerholm, A. Oral Immunization with a Salmonella enterica Serovar Typhi Vaccine Induces Specific Circulating Mucosa-Homing CD4 + and CD8 + T Cells in Humans Oral Immunization with a Salmonella enterica Serovar Typhi Vaccine Induces Specific Circulating Mucosa-Homing C. *Infect. Immun.* **70**, 5622–5627 (2002).
303. Salerno-Goncalves, R., Wahid, R. & Szein, M. B. Ex vivo kinetics of early and long-term multifunctional human leukocyte antigen E-specific CD8+ cells in volunteers immunized with the Ty21a typhoid vaccine. *Clin. Vaccine Immunol.* **17**, 1305–1314 (2010).
304. Yang, H. H. *et al.* An Outbreak of Typhoid Fever, Xing-An County, People's Republic of China, 1999: Estimation of the Field Effectiveness of Vi Polysaccharide Typhoid Vaccine. *J. Infect. Dis.* **183**, 1775–1780 (2001).
305. Keitel, W. A., Bond, N. L., Zahradnik, J. M., Cramton, T. A. & Robbins, J. B. Clinical and serological responses following primary and booster immunization with Salmonella typhi Vi capsular polysaccharide vaccines. *Vaccine* **12**, 195–199 (1994).
306. Bhaduri, B., Sc, M., Acosta, C. J., D, P. & Clemens, J. D. A Cluster-Randomized Effectiveness Trial of Vi Typhoid Vaccine in India. *N. Engl. J. Med.* 335–344 (2009).
307. Lin, F. Y., Ho, H. A. & Khiem, H. B. Salmonella typhi Vi conjugate vaccine reduced the incidence of typhoid fever in 2 to 5 year old children. *Evid. Based. Med.* **6**, 179 (2001).
308. Canh, D. G. *et al.* Effect of dosage on immunogenicity of a Vi conjugate vaccine injected twice into 2- to 5-year-old Vietnamese children. *Infect. Immun.* **72**, 6586–6588 (2004).
309. Micoli, F. *et al.* Vi-CRM197 as a new conjugate vaccine against Salmonella Typhi. *Vaccine* **29**, 712–720 (2011).
310. Bhutta, Z. A. *et al.* Immunogenicity and safety of the Vi-CRM 197 conjugate vaccine against typhoid fever in adults , children , and infants in south and southeast Asia : results from two randomised , observer-blind , age de-escalation , phase 2 trials. 119–129 (2014). doi:10.1016/S1473-3099(13)70241-X
311. Damme, P. Van *et al.* Safety , Immunogenicity and Dose Ranging of a New Vi- CRM 197 Conjugate Vaccine against Typhoid Fever : Randomized Clinical Testing in Healthy Adults. 1–7 (2011). doi:10.1371/journal.pone.0025398
312. Cui, C. *et al.* Physical and chemical characterization and immunologic properties of

- Salmonella enterica* serovar typhi capsular polysaccharide-diphtheria toxoid conjugates. *Clin. Vaccine Immunol.* **17**, 73–79 (2010).
313. Rosario, M. *et al.* Safety and immunogenicity of a Vi-DT typhoid conjugate vaccine : Phase I trial in Healthy Filipino adults and children. *Vaccine* **36**, 3794–3801 (2018).
  314. Simon, R. & Levine, M. M. Glycoconjugate vaccine strategies for protection against invasive *Salmonella* infections. *Hum. Vaccin. Immunother.* **8**, 494–498 (2012).
  315. Hohmann, E. L., Oletta, C. A., Killeen, K. P. & Miller, S. I. *phoP* / *phoQ*-Deleted *Salmonella typhi* ( Ty800 ) Is a Safe and Immunogenic Single-Dose Typhoid Fever Vaccine in Volunteers. *J. Infect. Dis.* **173**, 1408–1414 (1996).
  316. Tacket, C. O. & Levine, M. M. CVD 908, CVD 908-htrA, and CVD 909 Live Oral Typhoid Vaccines: A Logical Progression. *Clin. Infect. Dis.* **45**, S20–S23 (2007).
  317. Hindle, Z. *et al.* Characterization of *Salmonella enterica* derivatives harboring defined *aroC* and *Salmonella* pathogenicity island 2 type III secretion system (*ssaV*) mutations by immunization of healthy volunteers. *Infect. Immun.* **70**, 3457–67 (2002).
  318. Kirkpatrick, B. D. *et al.* Evaluation of *Salmonella enterica* serovar Typhi (Ty2 *aroC*-*ssaV*-) M01ZH09, with a defined mutation in the *Salmonella* pathogenicity island 2, as a live, oral typhoid vaccine in human volunteers. *Vaccine* **24**, 116–123 (2006).
  319. Tran, T. H. *et al.* A randomised trial evaluating the safety and immunogenicity of the novel single oral dose typhoid vaccine M01ZH09 in healthy Vietnamese children. *PLoS One* **5**, e11778 (2010).
  320. Tacket, C. O. *et al.* Comparison of the safety and immunogenicity of delta *aroC* delta *aroD* and delta *cya* delta *crp* *Salmonella typhi* strains in adult volunteers. *Infect. Immun.* **60**, 536–41 (1992).
  321. Tacket, C. O. *et al.* Clinical acceptability and immunogenicity of CVD 908 *Salmonella typhi* vaccine strain. *Vaccine* **10**, 443–446 (1992).
  322. Wang, J. Y., Noriega, F. R., Galen, J. E., Barry, E. & Levine, M. M. Constitutive expression of the Vi polysaccharide capsular antigen in attenuated *Salmonella enterica* serovar Typhi oral vaccine strain CVD 909. *Infect. Immun.* **68**, 4647–4652 (2000).
  323. Sahastrabudde, S., Carbis, R., Wierzbza, T. F. & Ochiai, R. L. Increasing rates of *Salmonella Paratyphi A* and the current status of its vaccine development. *Expert Rev. Vaccines* **12**,

- 1021–31 (2013).
324. Konadu, E., Shiloach, J., Bryla, D. A., Robbins, J. B. & Szu, S. C. Synthesis , Characterization , and Immunological Properties in Mice of Conjugates Composed of Detoxified Lipopolysaccharide of Salmonella paratyphi A Bound to Tetanus Toxoid , with Emphasis on the Role of O Acetyls. *Infect Immun* **64**, 2709–27143 (1996).
  325. Micoli, F. *et al.* O:2-CRM197 Conjugates against Salmonella Paratyphi A. *PLoS One* **7**, (2012).
  326. Kothari, S. *et al.* Purification of O-specific polysaccharide from lipopolysaccharide produced by Salmonella enterica serovar Paratyphi A. *Vaccine* **32**, 2457–2462 (2014).
  327. Gat, O. *et al.* Cell-Associated Flagella Enhance the Protection Conferred by Mucosally-Administered Attenuated Salmonella Paratyphi A Vaccines. *PLoS Negl. Trop. Dis.* **5**, e73 (2011).
  328. Galen, J. E. *et al.* Salmonella enterica serovar Typhi live vector vaccines finally come of age. *Immunol. Cell Biol.* **87**, 400–412 (2009).
  329. Gonzalez, C. *et al.* Salmonella typhi Vaccine Strain CVD 908 Expressing the Circumsporozoite Protein of Plasmodium falciparum: Strain Construction and Safety and Immunogenicity in Humans. *J. Infect. Dis.* **169**, 927–931 (1994).
  330. Tacket, C. O. *et al.* Safety and Immune Responses to Attenuated Salmonella enterica Serovar Typhi Oral Live Vector Vaccines Expressing Tetanus Toxin Fragment C. *Clin. Immunol.* **97**, 146–153 (2000).
  331. Khan, S. *et al.* Ability of SPI2 mutant of S. typhi to effectively induce antibody responses to the mucosal antigen enterotoxigenic E. coli heat labile toxin B subunit after oral delivery to humans. *Vaccine* **25**, 4175–4182 (2007).
  332. Frey, S. E. *et al.* A Phase I, dose-escalation trial in adults of three recombinant attenuated Salmonella Typhi vaccine vectors producing Streptococcus pneumoniae surface protein antigen PspA. *Vaccine* **31**, 4874–80 (2013).
  333. Tran Vu Thieu, N. *et al.* An evaluation of purified Salmonella Typhi protein antigens for the serological diagnosis of acute typhoid fever. *J. Infect.* **75**, 104–114 (2017).
  334. Maude, R. R. *et al.* A prospective study of the importance of enteric fever as a cause of non-malarial febrile illness in patients admitted to Chittagong Medical College Hospital ,

- Bangladesh. *BMC Infect. Dis.* **16**, 1–8 (2016).
335. Maude, R. R. *et al.* The diagnostic accuracy of three rapid diagnostic tests for typhoid fever at Chittagong Medical College Hospital, Chittagong, Bangladesh. *Trop. Med. Int. Health* **20**, 1376–84 (2015).
  336. Liang, L. *et al.* Immune profiling with a Salmonella Typhi antigen microarray identifies new diagnostic biomarkers of human typhoid. *Sci. Rep.* **3**, 1–10 (2013).
  337. Karkey, A. *et al.* Differential epidemiology of Salmonella Typhi and Paratyphi A in Kathmandu, Nepal: a matched case control investigation in a highly endemic enteric fever setting. *PLoS Negl. Trop. Dis.* **7**, e2391 (2013).
  338. Rondini, S. *et al.* Characterization of Citrobacter sp. line 328 as a source of Vi for a Vi-CRM197 glycoconjugate vaccine against Salmonella Typhi. *J. Infect. Dev. Ctries.* **6**, 763–773 (2012).
  339. Parkhill, J. *et al.* Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. *Nature* **413**, 848–52 (2001).
  340. Liang, L. *et al.* Immune profiling with a Salmonella Typhi antigen microarray identifies new diagnostic biomarkers of human typhoid. *Sci. Rep.* **3**, 1043 (2013).
  341. Ugalde, J. E., Gala, J. E. & Spano, S. Delivery of a Salmonella Typhi Exotoxin from a Host Intracellular Compartment. *Cell Host Microbe* **3**, 30–38 (2008).
  342. Langridge, G. C. *et al.* Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. *Genome Res.* **19**, 2308–2316 (2009).
  343. Langridge, G. C. *et al.* Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. *Genome Res.* **19**, 2308–16 (2009).
  344. R Core Team. R: A Language and Environment for Statistical Computing. *R Foundation for Statistical Computing Vienna Austria* **0**, {ISBN} 3-900051-07-0 (2014).
  345. Castro, N. C. & Azevedo, P. J. Significant motifs in time series. *Stat. Anal. Data Min.* (2012). doi:10.1002/sam.11134
  346. Shieh, J. iSAX: Indexing and Mining Terabyte Sized Time Series. *Work* (2008). doi:10.1145/1401890.1401966
  347. Hox, J. *Multilevel analysis - Techniques and applications. Multilevel analysis techniques and applications.* (2010).



348. Gelman, A. & Hill, J. *Data analysis using regression and multilevel/hierarchical models*. *Data Analysis Using Regression and Multilevel/Hierarchical Models* (2007).
349. Arthur, D. & Vassilvitskii, S. k-means++: the advantages of careful seeding. in *Proceedings of the eighteenth annual ACM-SIAM symposium on Discrete algorithms* (2007).  
doi:10.1145/1283383.1283494
350. Lloyd, S. P. Least Squares Quantization in PCM. *IEEE Trans. Inf. Theory* (1982).  
doi:10.1109/TIT.1982.1056489
351. Dembek, M. *et al.* High-throughput analysis of gene essentiality and sporulation in *Clostridium difficile*. *MBio* **6**, 1–13 (2015).
352. Barquist, L. *et al.* A comparison of dense transposon insertion libraries in the *Salmonella* serovars Typhi and Typhimurium. *Nucleic Acids Res.* **41**, 4549–4564 (2013).
353. Pham Thanh, D. *et al.* The Molecular and Spatial Epidemiology of Typhoid Fever in Rural Cambodia. *PLoS Negl. Trop. Dis.* **10**, e0004785 (2016).
354. Baker, S. *et al.* Combined high-resolution genotyping and geospatial analysis reveals modes of endemic urban typhoid fever transmission. *Open Biol.* **1**, 110008–110008 (2011).
355. Hendriksen, R. S. *et al.* Genomic dissection of travel-associated extended-spectrum-beta-lactamase-producing *Salmonella enterica* serovar typhi isolates originating from the Philippines: a one-off occurrence or a threat to effective treatment of typhoid fever? *J. Clin. Microbiol.* **53**, 677–80 (2015).
356. Baker, S., Hombach, J. & Marks, F. What Have We Learned From the Typhoid Fever Surveillance in Africa Program? *Clin. Infect. Dis.* **62**, S1–S3 (2016).
357. The World Health Organization. Epidemiological situation in Viet-Nam. *Twenty-first World Heal. Assem. agenda* **3 May**, (1968).
358. Greenberg, J. H. Public health problems relating to the Vietnam returnee. *JAMA* **207**, 697–702 (1969).
359. Levy, BS and Sidel, V. *War and public health*. (Oxford University Press, 2008).
360. Ochiai, R. L. *et al.* A study of typhoid fever in five Asian countries: disease burden and implications for controls. *Bull. World Health Organ.* **86**, 260–8 (2008).
361. ALI, M. *et al.* Geographic analysis of vaccine uptake in a cluster-randomized controlled trial in Hue, Vietnam. *Health Place* **13**, 577–587 (2007).

362. Cook, J. *et al.* The cost-effectiveness of typhoid Vi vaccination programs: Calculations for four urban sites in four Asian countries. *Vaccine* **26**, 6305–6316 (2008).
363. Cuong, N. Typhoid Vaccine Used in Vietnam and its Impact. *Consultation on Typhoid Vaccine Introduction and Typhoid Surveillance* (2011). Available at: [http://www.sabin.org/sites/sabin.org/files/CUONG Typhoid Vaccine in Vietnam.pdf](http://www.sabin.org/sites/sabin.org/files/CUONG%20Typhoid%20Vaccine%20in%20Vietnam.pdf).
364. Gordon, M. A. *et al.* Non-typhoidal salmonella bacteraemia among HIV-infected Malawian adults: high mortality and frequent recrudescence. *Aids* **16**, 1633–1641 (2002).
365. Le, T. *et al.* Epidemiology, seasonality, and predictors of outcome of AIDS-associated *Penicillium marneffei* infection in Ho Chi Minh City, Viet Nam. *Clin. Infect. Dis.* **52**, 945–52 (2011).
366. Phu Huong Lan, N. *et al.* Invasive Non-typhoidal Salmonella Infections in Asia: Clinical Observations, Disease Outcome and Dominant Serovars from an Infectious Disease Hospital in Vietnam. *PLoS Negl. Trop. Dis.* **10**, e0004857 (2016).
367. Ochiai, R. L. *et al.* Paratyphi A Rates, Asia. *Emerg. Infect. Dis.* **11**, 0–2 (2005).
368. Vlieghe, E. *et al.* Increase in Salmonella enterica serovar Paratyphi A infections in Phnom Penh, Cambodia, January 2011 to August 2013. *Euro Surveill.* **18**, (2013).
369. Chinh, N. T. *et al.* A randomized controlled comparison of azithromycin and ofloxacin for treatment of multidrug-resistant or nalidixic acid-resistant enteric fever. *Antimicrob. Agents Chemother.* **44**, 1855–9 (2000).
370. Hoa, N. T. *et al.* Community-acquired septicaemia in southern Viet Nam: the importance of multidrug-resistant Salmonella typhi. *Trans. R. Soc. Trop. Med. Hyg.* **92**, 503–8 (1998).
371. Holt, K. E. *et al.* Emergence of a globally dominant IncHI1 plasmid type associated with multiple drug resistant typhoid. *PLoS Negl. Trop. Dis.* **5**, e1245 (2011).
372. Wong, V. K. *et al.* An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. *Nat. Commun.* **7**, 12827 (2016).
373. Holt, K. E. *et al.* Temporal fluctuation of multidrug resistant salmonella typhi haplotypes in the mekong river delta region of Vietnam. *PLoS Negl. Trop. Dis.* **5**, e929 (2011).
374. Parry, C. M. *et al.* The Influence of reduced susceptibility to fluoroquinolones in Salmonella enterica serovar Typhi on the clinical response to ofloxacin therapy. *PLoS Negl. Trop. Dis.* **5**, 1–8 (2011).

375. Wong, V. K. *et al.* Phylogeographical analysis of the dominant multidrug-resistant H58 clade of *Salmonella* Typhi identifies inter- and intracontinental transmission events. *Nat. Genet.* **47**, 632–9 (2015).
376. Wain, J. *et al.* Quinolone-resistant *Salmonella* typhi in Viet Nam: molecular basis of resistance and clinical response to treatment. *Clin. Infect. Dis.* **25**, 1404–1410 (1997).
377. Connerton, P. *et al.* Epidemic typhoid in vietnam: molecular typing of multiple-antibiotic-resistant *Salmonella enterica* serotype typhi from four outbreaks. *J. Clin. Microbiol.* **38**, 895–7 (2000).
378. Pham Thanh, D. *et al.* A novel ciprofloxacin-resistant subclade of H58 *Salmonella* Typhi is associated with fluoroquinolone treatment failure. *Elife* **5**, (2016).
379. DeRoeck, D. *et al.* Typhoid vaccination: the Asian experience. *Expert Rev. Vaccines* **7**, 547–560 (2008).
380. Lin, F. Y. C. *et al.* The Efficacy of a *Salmonella typhi* Vi Conjugate Vaccine in Two-to-Five-Year-Old Children. *N. Engl. J. Med.* **344**, 1263–1269 (2001).
381. Vandemoortele, M. & Bird, K. *Viet Nam's progress on economic growth and poverty reduction : Impressive improvements. Overseas Development Institute* (2011).
382. IndexMundi. (2016). Available at: <http://www.indexmundi.com/facts/vietnam>. (Accessed: 13th January 2017)
383. Karkey, A. *et al.* The Ecological Dynamics of Fecal Contamination and *Salmonella* Typhi and *Salmonella* Paratyphi A in Municipal Kathmandu Drinking Water. *PLoS Negl. Trop. Dis.* **10**, e0004346 (2016).
384. UNICEF. Multiple Indicator Cluster Survey (MICS). *Statistics and Monitoring* Available at: [https://www.unicef.org/statistics/index\\_countrystats.html](https://www.unicef.org/statistics/index_countrystats.html). (Accessed: 13th January 2017)
385. The World Bank. World Bank Open Data - Vietnam. Available at: <http://data.worldbank.org/country/vietnam>. (Accessed: 13th January 2017)
386. Nguyen, Q. C. *et al.* A clinical, microbiological, and pathological study of intestinal perforation associated with typhoid fever. *Clin. Infect. Dis.* **39**, 61–7 (2004).
387. Khan, M. I. *et al.* Effectiveness of Vi capsular polysaccharide typhoid vaccine among children: A cluster randomized trial in Karachi, Pakistan. *Vaccine* **30**, 5389–5395 (2012).
388. Sur, D. *et al.* A Cluster-Randomized Effectiveness Trial of Vi Typhoid Vaccine in India. *N.*

- Engl. J. Med.* **361**, 335–344 (2009).
389. Crump, J. A. Building the case for wider use of typhoid vaccines. *Vaccine* **33**, C1–C2 (2015).
  390. Baker, S., Favorov, M. & Dougan, G. Searching for the elusive typhoid diagnostic. *BMC Infect. Dis.* **10**, 45 (2010).
  391. Parry, C. M., Wijedoru, L., Arjyal, A. & Baker, S. The utility of diagnostic tests for enteric fever in endemic locations. *Expert Rev. Anti. Infect. Ther.* **9**, 711–25 (2011).
  392. Gilman, R. H., Terminel, M., Levine, M. M., Hernandez-Mendoza, P. & Hornick, R. B. Relative efficacy of blood, urine, rectal swab, bone-marrow, and rose-spot cultures for recovery of *Salmonella typhi* in typhoid fever. *Lancet* **1**, 1211–3 (1975).
  393. Wain, J. *et al.* Quantitation of Bacteria in Bone Marrow from Patients with Typhoid Fever : Relationship between Counts and Clinical Features. *Society* **39**, 1571–1576 (2001).
  394. Waddington, C. S. *et al.* An Outpatient, Ambulant-Design, Controlled Human Infection Model Using Escalating Doses of *Salmonella Typhi* Challenge Delivered in Sodium Bicarbonate Solution. *Clin. Infect. Dis.* **58**, 1230–1240 (2014).
  395. Blohmke, C. J. *et al.* Interferon-driven alterations of the host's amino acid metabolism in the pathogenesis of typhoid fever. *J. Exp. Med.* **213**, 1061–77 (2016).
  396. Charles, R. C. *et al.* Immunoproteomic analysis of antibody in lymphocyte supernatant in patients with typhoid fever in Bangladesh. *Clin. Vaccine Immunol.* **21**, 280–285 (2014).
  397. Näsström, E. *et al.* *Salmonella Typhi* and *Salmonella Paratyphi A* elaborate distinct systemic metabolite signatures during enteric fever. *Elife* e03100 (2014). doi:10.7554/eLife.03100
  398. Bhutta, Z. A. Current concepts in the diagnosis and treatment of typhoid fever. *BMJ* **333**, 78–82 (2006).
  399. Arjyal, A. *et al.* Gatifloxacin versus ceftriaxone for uncomplicated enteric fever in Nepal: an open-label, two-centre, randomised controlled trial. *Lancet Infect. Dis.* **16**, 535–545 (2016).
  400. Wong, V. K. *et al.* Phylogeographical analysis of the dominant multidrug-resistant H58 clade of *Salmonella Typhi* identifies inter- and intracontinental transmission events. *Nat Genet* **47**, 632–639 (2015).
  401. Steele, A. D., Hay Burgess, D. C., Diaz, Z., Carey, M. E. & Zaidi, A. K. M. Challenges and Opportunities for Typhoid Fever Control: A Call for Coordinated Action. *Clin. Infect. Dis.* **62 Suppl 1**, S4-8 (2016).

402. Andrews, J. R. & Ryan, E. T. Diagnostics for invasive Salmonella infections: Current challenges and future directions. *Vaccine* **33 Suppl 3**, C8-15 (2015).
403. Sheikh, A. *et al.* Interferon- $\gamma$  and proliferation responses to Salmonella enterica Serotype Typhi proteins in patients with S. Typhi Bacteremia in Dhaka, Bangladesh. *PLoS Negl. Trop. Dis.* **5**, e1193 (2011).
404. Raffatellu, M. *et al.* The capsule encoding the *viaB* locus reduces interleukin-17 expression and mucosal innate responses in the bovine intestinal mucosa during infection with Salmonella enterica serotype Typhi. *Infect. Immun.* **75**, 4342–50 (2007).
405. Sheikh, A. *et al.* In vivo expression of Salmonella enterica serotype Typhi genes in the blood of patients with typhoid fever in Bangladesh. *PLoS Negl. Trop. Dis.* **5**, e1419 (2011).
406. Charles, R. C. *et al.* Identification of immunogenic Salmonella enterica serotype Typhi antigens expressed in chronic biliary carriers of S. Typhi in Kathmandu, Nepal. *PLoS Negl. Trop. Dis.* **7**, e2335 (2013).
407. Harris, J. B. *et al.* Identification of in vivo-induced bacterial protein antigens during human infection with Salmonella enterica serovar Typhi. *Infect. Immun.* **74**, 5161–8 (2006).
408. Moore, C. E. *et al.* Evaluation of the diagnostic accuracy of a typhoid IgM flow assay for the diagnosis of typhoid fever in Cambodian children using a Bayesian latent class model assuming an imperfect gold standard. *Am. J. Trop. Med. Hyg.* **90**, 114–20 (2014).
409. Pickard, D. *et al.* Composition , Acquisition , and Distribution of the Vi Exopolysaccharide-Encoding Salmonella enterica Pathogenicity Island SPI-7. *Society* **185**, 5055–5065 (2003).
410. Tsui, I. S. M., Yip, C. M. C., Hackett, J. & Morris, C. The type IVB pili of Salmonella enterica serovar Typhi bind to the cystic fibrosis transmembrane conductance regulator. *Infect. Immun.* **71**, 6049–50 (2003).
411. Faucher, S. P., Curtiss, R. & Daigle, F. Selective capture of Salmonella enterica serovar typhi genes expressed in macrophages that are absent from the Salmonella enterica serovar Typhimurium genome. *Infect. Immun.* **73**, 5217–21 (2005).
412. Zhang, X. *et al.* Salmonella enterica Serovar Typhi Uses Type IVB Pili To Enter Human Intestinal Epithelial Cells. *Society* **68**, 3067–3073 (2000).
413. Charles, R. C. *et al.* Characterization of anti-Salmonella enterica serotype Typhi antibody responses in bacteremic Bangladeshi patients by an immunoaffinity proteomics-based

- technology. *Clin. Vaccine Immunol.* **17**, 1188–95 (2010).
414. Tran Vu Thieu, N. *et al.* An evaluation of purified Salmonella Typhi protein antigens for the serological diagnosis of acute typhoid fever. *J. Infect.* **75**, 104–114 (2017).
  415. Fowler, C. C. *et al.* Emerging insights into the biology of typhoid toxin. *Curr. Opin. Microbiol.* **35**, 70–77 (2017).
  416. Martin, L. B. *et al.* Status of paratyphoid fever vaccine research and development. *Vaccine* **34**, 2900–2902 (2016).
  417. Luo, W., Friedman, M. S., Shedden, K., Hankenson, K. D. & Woolf, P. J. GAGE : generally applicable gene set enrichment for pathway analysis. *BMC Bioinformatics* **10**, 1–17 (2009).
  418. Kaniga, K., Bossio, J. C. & Galan, J. E. The Salmonella typhimurium invasion genes invF and invG encode homologues of the AraC and PulD family of proteins. *Mol. Microbiol.* **13**, 555–568 (1994).
  419. Collazo, C. M., Zierler, M. K. & Galan, J. E. Functional analysis of the Salmonella typhimurium invasion genes invI and invJ and identification of a target of the protein secretion apparatus encoded in the inv locus. *Mol. Microbiol.* **15**, 25–38 (1995).
  420. Darwin, K. H. & Miller, V. L. InvF is required for expression of genes encoding proteins secreted by the SPI1 type III secretion apparatus in Salmonella typhimurium. *J. Bacteriol.* **181**, 4949–4954 (1999).
  421. Gunn, J. S., Alpuche-Aranda, C. M., Loomis, W. P., Belden, W. J. & Miller, S. I. Characterization of the Salmonella typhimurium pagC/pagD chromosomal region. *J. Bacteriol.* **177**, 5040–5047 (1995).
  422. Zurawski, D. V. & Stein, M. A. SseA acts as the chaperone for the SseB component of the Salmonella Pathogenicity Island 2 translocon. *Mol. Microbiol.* **47**, 1341–1351 (2003).
  423. Schro, W. SseBCD Proteins Are Secreted by the Type III Secretion System of. *Microbiology* **183**, 6036–6045 (2001).
  424. Dufresne, K., Saulnier-Bellemare, J. & Daigle, F. Functional Analysis of the Chaperone-Usher Fimbrial Gene Clusters of Salmonella enterica serovar Typhi. *Front. Cell. Infect. Microbiol.* **8**, 1–13 (2018).
  425. Shi, W. & Sun, H. Type IV Pilus-Dependent Motility and Its Possible Role in Bacterial Pathogenesis. *Infect. Immun.* **70**, 1–4 (2002).

426. Craig, L., Pique, M. E. & Tainer, J. A. Type IV Pilus Structure and Pathogenicity. *Nat. Rev. Microbiol.* **2**, 363–378 (2004).
427. Fiedler, H. P., Krastel, P., Müller, J., Gebhardt, K. & Zeeck, A. Enterobactin: The characteristic catecholate siderophore of Enterobacteriaceae is produced by Streptomyces species. *FEMS Microbiol. Lett.* **196**, 147–151 (2001).
428. Nas, M. Y. & Cianciotto, N. P. Stenotrophomonas maltophilia produces an EntC-dependent catecholate siderophore that is distinct from enterobactin. *Microbiol. (United Kingdom)* **163**, 1590–1603 (2017).
429. Wilson, B. R., Bogdan, A. R., Miyazawa, M., Hashimoto, K. & Tsuji, Y. Siderophores in Iron Metabolism: From Mechanism to Therapy Potential. *Trends Mol. Med.* **22**, 1077–1090 (2016).
430. Anwar, E. *et al.* Vaccines for preventing typhoid fever ( Review ). (2014). doi:10.1002/14651858.CD001261.pub3. www.cochranelibrary.com
431. Boyd, M. A. *et al.* Serum bactericidal assays to evaluate typhoidal and nontyphoidal Salmonella vaccines. *Clin. Vaccine Immunol.* **21**, 712–721 (2014).
432. Nurjayadi, M. *et al.* Immunogenicity and Specificity of Anti recombinant Protein Fim-C-Salmonella typhimurium Antibody as a Model to Develop Typhoid Vaccine. *Procedia Chem.* **18**, 237–245 (2016).
433. Ghosh, S. *et al.* An adhesion protein of Salmonella enterica serovar Typhi is required for pathogenesis and potential target for vaccine development. *Proc. Natl. Acad. Sci. U. S. A.* **108**, 3348–53 (2011).
434. Das, S., Chowdhury, R., Ghosh, S. & Das, S. A recombinant protein of Salmonella Typhi induces humoral and cell-mediated immune responses including memory responses. *Vaccine* **35**, 4523–4531 (2017).
435. Moreno-Eutimio, M. a *et al.* Salmonella Typhi OmpS1 and OmpS2 porins are potent protective immunogens with adjuvant properties. *Immunology* **139**, 459–471 (2013).
436. Bumann, D. Identification of protective antigens for vaccination against systemic salmonellosis. *Front. Immunol.* **5**, 1–5 (2014).
437. Salerno-Gonçalves, R. *et al.* Use of a novel antigen expressing system to study the Salmonella enterica serovar Typhi protein recognition by T cells. *PLoS Negl. Trop. Dis.* **11**,

- 1–22 (2017).
438. Harris, J. B. *et al.* Identification of in vivo-induced bacterial protein antigens during human infection with *Salmonella enterica* serovar Typhi. *Infect. Immun.* **74**, 5161–5168 (2006).
  439. MacLennan, C. A. *et al.* The neglected role of antibody in protection against bacteremia caused by nontyphoidal strains of *Salmonella* in African children. *J. Clin. Invest.* **118**, 1553–1562 (2008).
  440. Ramachandran, G. *et al.* Opsonophagocytic Assay To Evaluate Immunogenicity of Nontyphoidal *Salmonella* Vaccines. *Clin. Vaccine Immunol.* **23**, 520–523 (2016).
  441. Donnelly, J. *et al.* Qualitative and quantitative assessment of meningococcal antigens to evaluate the potential strain coverage of protein-based vaccines. *Proc. Natl. Acad. Sci.* **107**, 19490–19495 (2010).
  442. Lindow, J. C., Fimlaid, K. A., Bunn, J. Y. & Kirkpatrick, B. D. Antibodies in action: Role of human opsonins in killing *Salmonella enterica* serovar Typhi. *Infect. Immun.* **79**, 3188–3194 (2011).
  443. Ciarlo, E. *et al.* Impact of the microbial derived short chain fatty acid propionate on host susceptibility to bacterial and fungal infections in vivo. *Sci. Rep.* **6**, 1–15 (2016).
  444. Fraser, A., Paul, M., Goldberg, E., Acosta, C. J. & Leibovici, L. Typhoid fever vaccines: Systematic review and meta-analysis of randomised controlled trials. *Vaccine* **25**, 7848–7857 (2007).
  445. von Rhein, C. *et al.* ClyA cytolysin from *Salmonella*: distribution within the genus, regulation of expression by SlyA, and pore-forming characteristics. *Int. J. Med. Microbiol.* **299**, 21–35 (2009).
  446. Fuentes, J. A., Villagra, N., Castillo-Ruiz, M. & Mora, G. C. The *Salmonella* Typhi hlyE gene plays a role in invasion of cultured epithelial cells and its functional transfer to *S. Typhimurium* promotes deep organ infection in mice. *Res. Microbiol.* **159**, 279–87 (2008).
  447. Karavolos, M. H. *et al.* *Salmonella* Typhi sense host neuroendocrine stress hormones and release the toxin haemolysin e. *EMBO Rep.* **12**, 252–258 (2011).
  448. Wai, S. N. *et al.* Vesicle-mediated export and assembly of pore-forming oligomers of the enterobacterial ClyA cytotoxin. *Cell* **115**, 25–35 (2003).
  449. Felgner, J. *et al.* *Development of ELISAs for diagnosis of acute typhoid fever in Nigerian*



- children. *PLoS neglected tropical diseases* **11**, (2017).
450. Mortimer, P. P. Mr N the milker, and Dr Koch's concept of the healthy carrier. *Lancet (London, England)* **353**, 1354–6 (1999).
  451. Franchi, L. Role of inflammasomes in Salmonella infection. *Front. Microbiol.* **2**, 1–6 (2011).
  452. Keuter, M. *et al.* Patterns of Proinflammatory Cytokines and Inhibitors during Typhoid Fever. *J. Infect. Dis.* **169**, 1306–1311 (1994).
  453. Raffatellu, M., Chessa, D., Wilson, R. P. & Ba, A. J. Capsule-Mediated Immune Evasion : a New Hypothesis Explaining Aspects of Typhoid Fever Pathogenesis. *Infect. Immun.* **74**, 19–27 (2006).
  454. Thompson, L. J. *et al.* Transcriptional response in the peripheral blood of patients infected with Salmonella enterica serovar Typhi. *Proc. Natl. Acad. Sci. U. S. A.* **106**, 22433–8 (2009).
  455. Bhutta, Z. A., Mansooralp, N. & Hussain, R. Plasma Cytokines in Paediatric Typhoidal Salmonellosis : Correlation with Clinical Course and Outcome. *J. Infect.* **35**, 253–256 (1997).
  456. Oswald, I. P., Wynn, T. A., Sher, A. & James, S. L. Interleukin 10 inhibits macrophage microbicidal activity by blocking the endogenous production of tumor necrosis factor alpha required as a costimulatory factor for interferon gamma-induced activation. *Proc. Natl. Acad. Sci. U. S. A.* **89**, 8676–80 (1992).
  457. Gupta, A. *et al.* Evaluation of community-based serologic screening for identification of chronic Salmonella typhi carriers in Vietnam. *Int. J. Infect. Dis.* **10**, 309–314 (2006).
  458. Thomason, B. M. & McWhorter, A. C. Rapid detection of typhoid carriers by means of fluorescent antibody techniques. *Bull. World Health Organ.* **33**, 681–685 (1965).
  459. Date, K. A., Bentsi-Enchill, A., Marks, F. & Fox, K. Typhoid fever vaccination strategies. *Vaccine* **33**, C55–C61 (2015).
  460. Ristori, C. *et al.* Investigation sobre el estado de portador de Salmonella Typhi-Paratyphi en pacientes intervenidos por patologia vesicular. *Bol Sanit Panam* **93**, 365–375 (1982).
  461. Levine, M. M., Black, R. E. & Lanata, C. Precise Estimation of the Numbers of Chronic Carriers of Salmonella typhi in Santiago , Chile , an Endemic Area. *J. Infect. Dis.* **146**, 724–726 (1982).
  462. Klemm, E. J. *et al.* Emergence of an Extensively Drug-Resistant Salmonella enterica Serovar Typhi Clone Harboring a Promiscuous Plasmid Encoding Resistance to Fluoroquinolones and

Third- Generation Cephalosporins. *MBio* **9**, 1–10 (2018).

463. Das, J. K. *et al.* Trends, associations, and antimicrobial resistance of salmonella typhi and paratyphi in Pakistan. *Am. J. Trop. Med. Hyg.* **99**, 48–54 (2018).

## Chapter 10 Appendices

### Appendix A Full list of *S. Typhi* CDSs identified following typhoid plasma challenge of the transposon mutant library in chapter 6

CDS_name	Products	Functional classes	logFC	logCPM	p.value	q.value	Time point	Other time points
STY1776	50S ribosomal subunit protein L35	3.A.2 Ribosomal protein synthesis, modification	11.7	6.9	1.3E-55	1.7E-54	D1	M3
STY3499	RNA polymerase sigma-54 factor (sigma-N)	2. Broad regulatory function	8.6	3.9	1.4E-11	2.5E-11	D1	M1M3
STY2668	pseudogene	4.A.3 Transport Carbohydrates, organic acids and alcohols	8.5	3.9	2.3E-11	4.2E-11	D1	M3
rpoH	RNA polymerase sigma-32 factor	2. Broad regulatory function	8.4	7.0	8.2E-56	1.1E-54	D1	
STY2001	hypothetical protein	5.H Hypothetical protein	7.9	3.3	1.2E-08	2.0E-08	D1	
STY3862	conserved hypothetical protein	5.H Hypothetical protein	7.2	2.8	8.4E-06	1.2E-05	D1	
STY3612	diaminopimelate epimerase	1.D.2 Aspartate Family	6.9	6.4	3.7E-41	2.2E-40	D1	
STY0012	DnaK protein (heat shock protein 70)	4.B Chaperones	6.1	7.6	2.1E-59	3.5E-58	D1	M3
STY3904	glucose inhibited division protein	3.A.7 DNA replication, repair, restriction, modification	5.9	7.2	1.4E-52	1.5E-51	D1	M1M3
STY2827	ribonuclease III	3.B.1 Degradation of RNA	5.6	5.7	3.0E-28	9.5E-28	D1	
tufB	elongation factor Tu	3.A.8 Protein translation and modification	5.6	8.1	1.1E-65	2.7E-64	D1	D8M1M3
STY4214	putative low-affinity inorganic phosphate transporter	4.A.5 Transport Anions	5.5	9.4	7.9E-83	5.4E-81	D1	M3
spiA	putative outer membrane secretory protein	4.I Pathogenesis	5.4	5.9	8.3E-30	2.8E-29	D1	M3
psd	phosphatidylserine decarboxylase proenzyme	3.A.11 Phospholipids	5.3	7.9	1.2E-58	1.9E-57	D1	
STY3914	ATP synthase epsilon subunit	1.A Degradation	5.2	5.3	1.3E-22	3.2E-22	D1	D8M3
STY1006	pseudogene	1.A Degradation	5.2	7.1	1.3E-45	9.4E-45	D1	M1
STY2958	hypothetical protein	5.H Hypothetical protein	5.1	8.5	6.0E-66	1.5E-64	D1	
yrfD	conserved hypothetical protein	5.H Hypothetical protein	5.1	9.4	1.0E-76	5.0E-75	D1	
STY3480	probable GTP-binding protein	5.I Unknown	5.1	7.9	8.1E-57	1.2E-55	D1	
STY0731	ferric uptake regulation protein	2. Broad regulatory function	5.0	5.9	1.5E-28	4.7E-28	D1	
trpS	tryptophanyl-tRNA synthetase	3.A.5 Aminoacyl tRNA synthesis, tRNA modification	4.9	8.1	6.2E-59	1.0E-57	D1	D8M1M3
trkA	potassium transport protein	4.A.2 Transport Cations	4.9	6.2	2.1E-31	7.5E-31	D1	M3
STY3937	thiophene and furan oxidation protein	4.G Detoxification	4.8	6.7	8.1E-38	3.9E-37	D1	M3
STY1909	peptidyl-tRNA hydrolase	3.A.5 Aminoacyl tRNA synthesis, tRNA modification	4.6	6.8	2.2E-38	1.1E-37	D1	
STY1357	peptide transport system permease protein SapC	4.A.6 Transport Other	4.6	4.3	6.6E-12	1.2E-11	D1	M1M3
STY2568	phosphate acetyltransferase	1.A.1 Degradation of carbohydrate	4.6	4.3	6.6E-12	1.2E-11	D1	M3
STY3809	6-phosphofructokinase	1.B.1 Glycolysis	4.6	9.5	1.3E-68	3.6E-67	D1	M1M3
safA	probable lipoprotein	4.I Pathogenesis	4.6	6.4	6.8E-33	2.6E-32	D1	

wcaE	putative glycosyltransferase	3.C.2 Surface polysacchar	4.6	8.1	2.8E-54	3.4E-53	D1	
STY4629	hypothetical protein	4.I Pathogenesis	4.4	4.6	6.7E-14	1.3E-13	D1	
ptsJ	putative transcriptional regulator	2. Broad regulatory function	4.4	6.2	3.0E-29	9.8E-29	D1	M1M3
STY0107	survival protein SurA precursor	3.A.8 Protein translation and modification	4.1	8.7	3.7E-54	4.5E-53	D1	M3
secG	protein-export membrane protein	4.A Transport/binding proteins	4.1	7.5	4.1E-41	2.4E-40	D1	
ssrB	putative two-component response regulator	4.I Pathogenesis	4.0	6.6	5.3E-32	2.0E-31	D1	
STY1744	pyruvate kinase	1.B.1 Glycolysis	3.9	5.1	1.5E-16	3.1E-16	D1	M3
STY4231	putative lipoprotein	3.C.1 Membranes lipoprotein	3.9	10.0	5.1E-58	7.9E-57	D1	
STY0781	succinyl-CoA synthetase beta chain	1.B.3 Tricarboxylic acid cycle	3.8	6.9	2.1E-32	7.9E-32	D1	
STY4681	putative transcriptional regulator	2. Broad regulatory function	3.8	9.2	1.6E-51	1.7E-50	D1	
crcB	putative membrane protein	3.C.1 Membranes lipoprotein	3.8	7.3	8.8E-37	4.1E-36	D1	M1M3
STY0181	aconitate hydratase 2 (citrate hydro-lyase 2)	1.B.3 Tricarboxylic acid cycle	3.8	7.0	1.4E-33	5.6E-33	D1	M3
STY0599	hypothetical protein	5.I Unknown	3.8	7.2	8.2E-35	3.4E-34	D1	M3
STY4594	hypothetical protein	4.I Pathogenesis	3.8	10.4	7.4E-56	1.0E-54	D1	D8
STY0374	possible transmembrane regulator	2. Broad regulatory function	3.7	7.8	7.9E-40	4.2E-39	D1	
STY4808	conserved hypothetical protein	5.H Hypothetical protein	3.7	8.8	2.1E-47	1.7E-46	D1	
STY3911	ATP synthase alpha subunit	1.A Degradation	3.7	5.7	1.6E-20	3.9E-20	D1	
STY3092	hypothetical protein	5.I Unknown	3.7	8.4	4.1E-44	2.8E-43	D1	
STY1378	transcriptional regulatory protein TyrR	2. Broad regulatory function	3.6	8.9	8.4E-46	6.4E-45	D1	
STY4842	probable regulatory protein	4.I Pathogenesis	3.6	7.4	5.8E-35	2.4E-34	D1	D8
STY3463	polynucleotide phosphorylase	3.A.9 RNA synthesis, modification	3.6	11.4	2.6E-53	2.9E-52	D1	D8M1M3
STY4615	putative phage baseplate assembly protein	4.I Pathogenesis	3.5	7.7	3.8E-36	1.7E-35	D1	
STY2790	conserved hypothetical protein	5.H Hypothetical protein	3.5	8.2	1.3E-39	6.9E-39	D1	M3
STY1463	putative membrane protein	3.C.1 Membranes lipoprotein	3.5	7.3	7.1E-33	2.7E-32	D1	
STY4875	putative membrane protein	3.C.1 Membranes lipoprotein	3.4	10.1	4.2E-48	3.6E-47	D1	
STY2622	conserved hypothetical protein	5.H Hypothetical protein	3.4	9.5	9.4E-46	7.0E-45	D1	
STY2532	putative membrane protein	3.C.1 Membranes lipoprotein	3.4	8.3	1.6E-39	8.3E-39	D1	
STY3689	phage baseplate assembly protein	5.A Laterally acquired elements	3.4	8.6	3.4E-41	2.0E-40	D1	
malX	pseudogene	4.A.3 Transport Carbohydes, organic acids and alcohols	3.4	6.5	1.7E-25	4.8E-25	D1	
STY1918	hydrogenase-1 operon protein HyaF	1.B.7.a Aerobic Respiration	3.4	8.6	9.6E-41	5.4E-40	D1	
tufA	elongation factor Tu	3.A.2 Ribosomal protein synthesis, modification	3.4	6.2	1.5E-22	3.8E-22	D1	M1
STY3907	ATP synthase A chain	1.A Degradation	3.4	5.0	1.7E-13	3.2E-13	D1	
cl	phage immunity repressor protein	5.A Laterally acquired elements	3.4	8.3	2.1E-38	1.1E-37	D1	
STY2829	GTP-binding protein LepA	3.A.8 Protein translation and modification	3.4	8.8	5.7E-41	3.3E-40	D1	D8
STY1162	PhoH protein (phosphate starvation-inducible protein PsiH)	1.C Central Intermediary metabolism	3.3	9.5	3.8E-44	2.6E-43	D1	
steA	pseudogene	3.C.3 Surface structure	3.3	8.8	8.3E-41	4.7E-40	D1	M3

STY1099	putative secreted protein	3.C.1 Membranes lipoprotein	3.3	8.8	8.3E-41	4.7E-40	D1	
STY3392	conserved hypothetical protein	5.H Hypothetical protein	3.3	8.9	2.2E-41	1.3E-40	D1	
STY2086	conserved hypothetical protein	5.H Hypothetical protein	3.3	9.0	1.5E-41	8.8E-41	D1	
STY1218	flagellar basal-body rod protein FlgG (distal rod protein)	3.C.3 Surface structure	3.3	9.2	2.3E-42	1.4E-41	D1	
STY0514	putative membrane protein	3.C.1 Membranes lipoprotein	3.3	9.3	2.9E-42	1.8E-41	D1	
STY0103	bis(5'-nucleosyl)-tetrakisphosphate	1.F.4 Salvage of nucleosides and nucleotides	3.3	5.1	7.8E-14	1.5E-13	D1	M1M3
sseB	putative pathogenicity island effector protein	4.I Pathogenesis	3.3	8.5	4.5E-38	2.2E-37	D1	M3
STY3460	probable amino acid permease	4.A.1 Transport amino acid and amines	3.2	10.1	6.9E-44	4.7E-43	D1	M3
STY4438	putative exported protein	3.C.1 Membranes lipoprotein	3.2	9.9	5.2E-43	3.3E-42	D1	
STY3952	Solute binding receptor protein	1.B.7.b Anaerobic Respiration	3.2	8.6	1.1E-37	5.3E-37	D1	
STY3626	probable UDP-N-acetyl-D-mannosaminuronic acid transferase	3.C.2 Surface polysacchar	3.2	8.5	3.0E-37	1.4E-36	D1	
STY1248	conserved hypothetical protein	5.H Hypothetical protein	3.2	9.0	2.0E-39	1.0E-38	D1	
pduV	putative propanediol utilization protein PduV	1.A.1 Degradation of carbohydrate	3.2	8.5	1.0E-36	4.7E-36	D1	
STY3778	heat shock protein	3.B.3 Degradation of proteins, peptides, glycoproteins	3.2	9.9	5.7E-42	3.5E-41	D1	
STY1653	fumarate hydratase class II	1.B.3 Tricarboxylic acid cycle	3.2	6.8	5.7E-25	1.6E-24	D1	
STY0192	hypoxanthine phosphoribosyltransferase	1.F.4 Salvage of nucleosides and nucleotides	3.2	8.2	9.9E-35	4.1E-34	D1	
STY0186	putative transcriptional regulator	2. Broad regulatory function	3.1	9.5	3.4E-40	1.9E-39	D1	D8
STY3547	probable LysR-family transcriptional regulator	2. Broad regulatory function	3.1	9.6	4.4E-40	2.4E-39	D1	
STY1251	NADH dehydrogenase	1.B.7.a Aerobic Respiration	3.1	6.4	8.7E-22	2.2E-21	D1	M1M3
STY1561	spermidine N1-acetyltransferase	1.E Polyamine synthesis	3.1	9.5	2.7E-39	1.4E-38	D1	
STY3633	UDP-N-acetylglucosamine epimerase (UDP-GlcNAc-2-epimerase)	3.C.2 Surface polysacchar	3.1	9.6	2.7E-39	1.4E-38	D1	
STY2878	putative type I secretion protein	4.I Pathogenesis	3.1	10.0	7.3E-40	3.9E-39	D1	
STY2751	GMP synthase (glutamine-hydrolyzing)	1.F.1 Purine ribonucleotide biosynthesis	3.0	5.9	1.2E-17	2.5E-17	D1	M1M3
STY2836	ATP-dependent RNA helicase SrmB	3.A.9 RNA synthesis, modification	3.0	7.9	3.2E-31	1.1E-30	D1	D8M3
STY1372	phage shock protein B	5.F Adaptions and atypical conditions	3.0	5.7	2.6E-16	5.3E-16	D1	
STY1602	hypothetical protein	5.I Unknown	3.0	7.1	1.6E-25	4.5E-25	D1	
STY3816	manganese superoxide dismutase	4.G Detoxification	3.0	6.5	7.2E-22	1.8E-21	D1	
STY0447	conserved hypothetical protein	5.H Hypothetical protein	3.0	8.5	1.1E-33	4.3E-33	D1	
menC	O-succinylbenzoate-CoA synthase	1.G.11 Menaquinone	3.0	8.7	1.5E-34	6.3E-34	D1	
hyaC2	Ni/Fe-hydrogenase 1 b-type cytochrome subunit HyaC2	1.B.7.a Aerobic Respiration	3.0	8.6	3.1E-34	1.3E-33	D1	
STY2839	conserved hypothetical protein	5.H Hypothetical protein	3.0	8.3	2.8E-32	1.0E-31	D1	D8
STY4497	alpha-galactosidase	1.A.1 Degradation of carbohydrate	3.0	9.3	1.6E-36	7.4E-36	D1	
STY2739	uracil phosphoribosyltransferase	1.F.4 Salvage of nucleosides and nucleotides	3.0	8.9	4.1E-35	1.7E-34	D1	M3
STY1937	conserved hypothetical protein	5.H Hypothetical protein	3.0	8.8	4.4E-34	1.8E-33	D1	
STY3507	aerobic respiration control sensor protein	2. Broad regulatory function	2.9	8.2	3.1E-31	1.1E-30	D1	

STY2689	putative membrane protein	3.C.1 Membranes lipoprotein	2.9	8.8	8.5E-34	3.4E-33	D1	
yjga	conserved hypothetical protein	5.H Hypothetical protein	2.9	6.6	2.0E-21	4.9E-21	D1	M1M3
STY1569	conserved hypothetical protein	5.H Hypothetical protein	2.9	8.1	1.2E-30	4.1E-30	D1	
STY0970	hypothetical protein	5.I Unknown	2.9	5.9	3.0E-17	6.4E-17	D1	M1M3
STY2145	tyrosine-specific transport protein	4.A.1 Transport amino acid and amines	2.9	7.6	1.2E-27	3.6E-27	D1	D8M3
STY0893	conserved hypothetical protein	5.H Hypothetical protein	2.9	7.8	8.5E-29	2.8E-28	D1	
STY0516	haemolysin expression modulating protein	2. Broad regulatory function	2.9	5.7	3.2E-15	6.5E-15	D1	M1M3
STY1608	hypothetical protein	5.I Unknown	2.9	8.5	1.1E-31	3.9E-31	D1	M3
sigD	cell invasion protein	4.I Pathogenesis	2.9	8.7	1.7E-32	6.3E-32	D1	
STY1033	putative bacteriophage protein	5.A Laterally acquired elements	2.9	8.9	8.0E-33	3.1E-32	D1	
STY4410	putative pseudouridine synthase	3.A.3 Ribosomal maturation and modification	2.9	10.2	2.4E-36	1.1E-35	D1	
ttrC	tetrathionate reductase subunit C (membrane protein)	4.I Pathogenesis	2.9	8.7	3.0E-32	1.1E-31	D1	
eutS	putative ethanolamine utilization protein EutS	#N/A	2.9	7.3	3.6E-25	1.0E-24	D1	
STY2681	thiosulphate-binding protein precursor	4.A.5 Transport Anions	2.8	8.2	1.2E-29	4.1E-29	D1	
asrB	anaerobic sulfite reductase subunit B	1.B.7.b Anaerobic Respiration	2.8	8.2	5.2E-29	1.7E-28	D1	
STY4121	putative hexulose-6-phosphate synthase	1.C Central Intermediary metabolism	2.8	8.6	1.3E-30	4.6E-30	D1	
STY2504	pseudogene	2. Broad regulatory function	2.8	7.6	4.7E-26	1.4E-25	D1	
STY2851	ftsH suppressor protein SfhB	3.A.2 Ribosomal protein synthesis, modification	2.8	9.9	2.9E-34	1.2E-33	D1	D8M3
STY3845	putative CopG-family DNA-binding protein	5.I Unknown	2.8	8.0	4.9E-28	1.5E-27	D1	
STY2320	GDP-fucose synthetase	1.C.3 Sugar Nucleotides	2.8	9.4	1.5E-32	5.7E-32	D1	
STY1501	putative secreted protein	3.C.1 Membranes lipoprotein	2.8	8.3	1.0E-28	3.3E-28	D1	D8
STY1908	putative membrane protein	3.C.1 Membranes lipoprotein	2.8	8.6	6.3E-30	2.2E-29	D1	
STY0450	hypothetical protein	5.I Unknown	2.7	7.2	5.2E-23	1.3E-22	D1	
bcfA	fimbrial subunit	3.C.3 Surface structure	2.7	9.2	1.5E-31	5.4E-31	D1	D8
gph	phosphoglycolate phosphatase	1.A.1 Degradation of carbohydrate	2.7	10.3	6.1E-34	2.5E-33	D1	
STY1262	spermidine/putrescine-binding periplasmic protein precursor	4.A.1 Transport amino acid and amines	2.7	8.8	1.9E-30	6.6E-30	D1	
STY3441	PTS system, galactitol-specific IIA component	4.A.3 Transport Carbohydrates, organic acids and alcohols	2.7	8.4	1.3E-28	4.3E-28	D1	
STY1758	conserved hypothetical FAD-binding protein	5.I Unknown	2.7	10.1	2.3E-33	9.1E-33	D1	
stgB	fimbrial chaperone protein	3.C.3 Surface structure	2.7	8.5	4.4E-29	1.4E-28	D1	
STY1076	conserved hypothetical protein	5.H Hypothetical protein	2.7	5.7	3.4E-14	6.5E-14	D1	
STY2697	putative membrane protein	3.C.1 Membranes lipoprotein	2.7	8.4	1.8E-28	5.8E-28	D1	
yacK	possible multicopper oxidase precursor	5.I Unknown	2.7	10.1	7.2E-33	2.8E-32	D1	
STY1563	pseudogene	5.H Hypothetical protein	2.7	6.8	1.2E-20	3.0E-20	D1	
STY0910	putrescine-binding periplasmic protein precursor	4.A.1 Transport amino acid and amines	2.7	8.6	9.2E-29	3.0E-28	D1	
STY4119	putative sugar isomerase	5.I Unknown	2.7	9.0	6.4E-30	2.2E-29	D1	
STY2728	glycine cleavage system transcriptional repressor	2. Broad regulatory function	2.7	9.2	1.6E-30	5.6E-30	D1	

STY4481	NrfG protein	3.C.1 Membranes lipoprotein	2.7	9.2	2.9E-30	1.0E-29	D1	
STY0006	putative amino-acid transport protein	4.A Transport/binding proteins	2.7	9.8	1.3E-31	5.0E-31	D1	
STY1573	putative ABC transporter ATP/GTP-binding protein	4.A Transport/binding proteins	2.7	8.1	1.7E-26	5.2E-26	D1	
STY3199	thiol:disulfide interchange protein	3.A.8 Protein translation and modification	2.7	9.1	9.2E-30	3.2E-29	D1	D8
STY2970	formate hydrogenlyase subunit 6	1.B.8 Fermentation	2.7	7.7	7.7E-25	2.1E-24	D1	
STY0393	probable terminal oxidase subunit II	1.B.7.c Electron Transport	2.7	9.4	2.4E-30	8.5E-30	D1	
STY4018	conserved hypothetical protein	5.H Hypothetical protein	2.7	9.1	9.8E-30	3.3E-29	D1	
STY3665	hypothetical protein	5.I Unknown	2.6	8.2	1.1E-26	3.4E-26	D1	
pduS	putative ferredoxin	1.B.7.c Electron Transport	2.6	8.5	1.9E-27	6.0E-27	D1	M3
ssaL	putative secretion system protein	4.I Pathogenesis	2.6	6.1	1.0E-15	2.1E-15	D1	
STY4611	probable phage tail fibre protein	4.I Pathogenesis	2.6	10.3	1.6E-31	5.9E-31	D1	
STY0194	hypothetical ABC transporter ATP-binding protein	4.A Transport/binding proteins	2.6	9.6	7.1E-30	2.5E-29	D1	D8
trkH	trk system potassium uptake protein	1.C.5 Sulphur Metabolism	2.6	7.4	2.3E-22	5.8E-22	D1	D8M3
STY0865	putative membrane protein	3.C.1 Membranes lipoprotein	2.6	9.6	1.1E-29	3.7E-29	D1	D8
STY1439	putative membrane protein	3.C.1 Membranes lipoprotein	2.6	9.0	2.4E-28	7.7E-28	D1	
STY1016	exonuclease	5.A Laterally acquired elements	2.6	7.9	2.8E-24	7.6E-24	D1	D8
sgaH	putative hexulose-6-phosphate synthase	1.C Central Intermediary metabolism	2.6	9.0	8.0E-28	2.5E-27	D1	
celB	PTS system, cellobiose-specific IIC component	4.A.3 Transport Carbohydrates, organic acids and alcohols	2.6	8.7	7.9E-27	2.4E-26	D1	
STY2184	flagellar motor switch protein FliM	3.C.3 Surface structure	2.6	8.6	1.1E-26	3.2E-26	D1	
cysQ	cysQ protein	1.C.5 Sulphur Metabolism	2.6	8.3	1.0E-25	2.9E-25	D1	
STY3946	putative membrane transport protein	4.A Transport/binding proteins	2.5	9.8	3.0E-29	1.0E-28	D1	
STY2901	pseudogene	4.A Transport/binding proteins	2.5	10.2	6.1E-30	2.1E-29	D1	
STY1790	conserved hypothetical protein	5.H Hypothetical protein	2.5	8.4	9.4E-26	2.8E-25	D1	
safD	putative fimbrial structural subunit	4.I Pathogenesis	2.5	7.2	8.0E-21	1.9E-20	D1	
STY3307	D-mannan oxidoreductase	1.A.1 Degradation of carbohydrate	2.5	9.4	3.3E-28	1.1E-27	D1	D8
STY3122	regulatory protein for glycine cleavage pathway	1.C Central Intermediary metabolism	2.5	9.1	2.5E-27	7.8E-27	D1	
STY0395	AraC-family transcriptional regulator	2. Broad regulatory function	2.5	8.9	6.5E-27	2.0E-26	D1	D8
STY0479	probable secreted protein	3.C.1 Membranes lipoprotein	2.5	6.8	3.7E-18	8.2E-18	D1	M3
STY0134	probable activator protein in leuABCD operon	1.D.6 Pyruvate Family	2.5	7.3	1.3E-20	3.2E-20	D1	M3
phnU	probable membrane component of 2-aminoethylphosphonate transporter	4.A.1 Transport amino acid and amines	2.5	8.6	1.2E-25	3.6E-25	D1	
STY4755	putative exported protein	3.C.1 Membranes lipoprotein	2.5	10.2	8.9E-29	2.9E-28	D1	
STY0887	hypothetical ABC transporter ATP-binding protein	4.A Transport/binding proteins	2.5	9.3	5.0E-27	1.5E-26	D1	
STY2832	sigma-E factor negative regulatory protein	2. Broad regulatory function	2.5	5.6	2.9E-12	5.4E-12	D1	
STY0982	integration host factor beta-subunit (IHf-beta)	3.A.7 DNA replication, repair, restriction, modification	2.5	9.4	3.3E-27	1.0E-26	D1	D8M3
STY2595	DedD protein	5.I Unknown	2.5	7.5	4.9E-21	1.2E-20	D1	
STY0007	transaldolase B	1.B.5.b Non-oxydative branch	2.5	9.2	1.8E-26	5.4E-26	D1	

glpD	aerobic glycerol-3-phosphate dehydrogenase	1.B.7.a Aerobic Respiration	2.5	8.5	1.9E-24	5.1E-24	D1	
sipB	pathogenicity island 1 effector protein	4.I Pathogenesis	2.5	9.5	6.4E-27	1.9E-26	D1	
STY2937	glycine betaine-binding periplasmic protein precursor	4.A.1 Transport amino acid and amines	2.5	9.8	1.9E-27	6.0E-27	D1	
stpA	tyrosine phosphatase (associated with virulence)	3.A.8 Protein translation and modification	2.4	8.2	1.2E-23	3.2E-23	D1	
spak	secretory protein (associated with virulence)	4.I Pathogenesis	2.4	7.4	2.1E-20	5.1E-20	D1	
STY1871	putative heat shock protein	4.B Chaperones	2.4	8.8	4.1E-25	1.2E-24	D1	
STY1153	glucose-1-phosphatase precursor (G1Pase), secreted	1.C Central Intermediary metabolism	2.4	9.6	1.4E-26	4.3E-26	D1	
STY3684	putative regulatory protein	5.I Unknown	2.4	8.9	3.1E-25	8.7E-25	D1	D8
STY0475	hpothetical major facilitator family transport protein	4.A Transport/binding proteins	2.4	8.9	3.4E-25	9.6E-25	D1	
phnS	probable periplasmic binding component of 2-aminoethylphosphonate transporter	4.A.1 Transport amino acid and amines	2.4	8.8	9.5E-25	2.6E-24	D1	
STY0383	probable lipoprotein	3.C.1 Membranes lipoprotein	2.4	8.7	1.9E-24	5.1E-24	D1	
STY3491	putative membrane protein	3.C.1 Membranes lipoprotein	2.4	9.4	5.3E-26	1.6E-25	D1	
STY2820	putative transmembrane transport protein	4.A Transport/binding proteins	2.4	6.5	6.9E-16	1.4E-15	D1	
STY0814	molybdate-binding periplasmic protein precursor	4.A.2 Transport Cations	2.4	8.5	6.5E-24	1.8E-23	D1	
STY3905	glucose inhibited division protein	3.A.7 DNA replication, repair, restriction, modification	2.4	9.2	2.0E-25	5.8E-25	D1	
STY3208	hypothetical protein	5.I Unknown	2.4	8.3	3.9E-23	1.0E-22	D1	
sthB	putative fimbrial chaperone protein	3.C.3 Surface structure	2.4	9.2	2.5E-25	7.1E-25	D1	
STY2650	nucleoside permease NupC	4.A.6 Transport Other	2.4	9.4	1.0E-25	3.0E-25	D1	D8M3
STY3384	bacitracin resistance protein (putative undecaprenol kinase)	5.D Drug/Analogue sensitivity	2.4	9.4	1.3E-25	3.8E-25	D1	
STY1091	outer membrane protein A	3.C Cell envelope	2.4	8.0	5.7E-22	1.4E-21	D1	
mtgA	monofunctional biosynthetic peptidoglycan transglycosylase	3.C.4 Murein sacculus and peptidoglycan	2.4	9.2	4.3E-25	1.2E-24	D1	D8
STY2562	putative oxetanocin A biosynthetic enzyme	5.I Unknown	2.4	8.4	7.3E-23	1.9E-22	D1	
menB	naphthoate synthase	1.G.11 Menaquinone	2.4	8.9	3.9E-24	1.0E-23	D1	
STY2367	putative membrane protein	3.C.1 Membranes lipoprotein	2.3	10.0	6.0E-26	1.8E-25	D1	D8
STY3590	putative membrane protein	3.C.1 Membranes lipoprotein	2.3	9.6	2.8E-25	7.9E-25	D1	D8
pduT	putative propanediol utilization protein PduT	1.A.1 Degradation of carbohydrate	2.3	7.9	6.5E-21	1.6E-20	D1	
STY3804	putative carbohydrate kinase	5.I Unknown	2.3	9.2	2.1E-24	5.7E-24	D1	
STY0909	conserved hypothetical protein	5.H Hypothetical protein	2.3	7.5	2.8E-19	6.4E-19	D1	
STY2971	formate hydrogenlyase subunit 5	1.B.8 Fermentation	2.3	9.4	8.3E-25	2.3E-24	D1	
STY2571	putative transketolase N-terminal section	1.C Central Intermediary metabolism	2.3	8.4	1.7E-22	4.3E-22	D1	
invA	possible secretory protein (associated with virulence)	4.I Pathogenesis	2.3	8.9	1.1E-23	3.0E-23	D1	D8
STY1629	putative bacteriophage protein	5.A Laterally acquired elements	2.3	9.4	2.1E-24	5.8E-24	D1	
STY0211	dosage-dependent dnaK suppressor protein	3.A.7 DNA replication, repair, restriction, modification	2.3	7.6	1.5E-19	3.4E-19	D1	M3
STY1938	conserved hypothetical protein	5.H Hypothetical protein	2.3	7.5	5.3E-19	1.2E-18	D1	
STY1365	pseudogene	5.I Unknown	2.3	8.1	5.4E-21	1.3E-20	D1	



STY2613	pseudogene	5.H Hypothetical protein	2.3	8.6	1.6E-22	4.1E-22	D1	D8
yfbB	conserved hypothetical protein	5.H Hypothetical protein	2.3	8.3	1.5E-21	3.8E-21	D1	
STY4415	putative membrane protein	3.C.1 Membranes lipoprotein	2.3	8.9	4.0E-23	1.0E-22	D1	
STY2343	putative two-component system sensor kinase	2. Broad regulatory function	2.3	9.3	6.6E-24	1.8E-23	D1	
STY2811	putative sensor kinase protein	2. Broad regulatory function	2.3	8.6	2.2E-22	5.5E-22	D1	
STY3677	major capsid protein	5.A Laterally acquired elements	2.3	9.9	1.3E-24	3.5E-24	D1	
STY0726	putative lipoprotein	3.C.1 Membranes lipoprotein	2.3	7.1	2.8E-17	6.0E-17	D1	
STY1759	putative membrane protein	3.C.1 Membranes lipoprotein	2.3	8.8	1.2E-22	3.1E-22	D1	
STY2290	6-phosphogluconate dehydrogenase, decarboxylating	1.B.5 Pentose phosphate	2.3	9.4	9.6E-24	2.6E-23	D1	M1
STY3823	L rhamnose-proton symporter	4.A.3 Transport Carbohydrates, organic acids and alcohols	2.3	9.6	6.2E-24	1.7E-23	D1	
STY2128	methyl-accepting chemotaxis protein II	4.D Chemotaxis and mobility	2.3	9.0	6.7E-23	1.7E-22	D1	M3
ybdQ	conserved hypothetical protein	5.H Hypothetical protein	2.3	8.3	4.1E-21	1.0E-20	D1	
STY0653	alkyl hydroperoxide reductase c22 protein	4.G Detoxification	2.3	6.9	2.7E-16	5.6E-16	D1	
nrdI	NrdI protein	1.F.5 Miscellaneous	2.3	7.7	4.3E-19	9.8E-19	D1	
STY2977	hydrogenase isoenzymes formation protein HypB	1.B.8 Fermentation	2.3	9.6	1.4E-23	3.7E-23	D1	D8
STY0321	Rhs-family protein	4.I Pathogenesis	2.3	10.3	2.1E-24	5.8E-24	D1	
STY4203	pseudogene	1.A.2 Degradation of amino acids	2.2	7.8	1.9E-19	4.4E-19	D1	M3
STY2137	trehalose-6-phosphate synthase	5.F Adaptions and atypical conditions	2.2	9.8	9.7E-24	2.6E-23	D1	
STY3632	glucose-1-phosphate thymidyltransferase	3.C.2 Surface polysacchar	2.2	9.1	1.7E-22	4.2E-22	D1	
pyrB	aspartate carbamoyltransferase catalytic subunit	1.F.2 Pyrimidine ribonucle	2.2	9.2	8.3E-23	2.1E-22	D1	
yjiA	putative secreted protein	3.C.1 Membranes lipoprotein	2.2	8.7	1.0E-21	2.6E-21	D1	
STY2021	putative bacteriophage protein	5.A Laterally acquired elements	2.2	8.4	9.2E-21	2.2E-20	D1	
STY3402	conserved hypothetical protein	5.H Hypothetical protein	2.2	8.5	5.4E-21	1.3E-20	D1	
STY4603	hypothetical protein	4.I Pathogenesis	2.2	11.0	1.4E-24	3.8E-24	D1	
STY3707	conserved hypothetical protein	5.H Hypothetical protein	2.2	9.7	5.0E-23	1.3E-22	D1	
STY2583	histidine transport system permease protein	4.A.1 Transport amino acid and amines	2.2	8.2	6.3E-20	1.5E-19	D1	
STY4206	conserved hypothetical protein	5.H Hypothetical protein	2.2	8.6	5.7E-21	1.4E-20	D1	D8
STY3764	putative serine hydroxymethyltransferase	5.I Unknown	2.2	8.7	3.8E-21	9.4E-21	D1	
STY4127	putative membrane protein	3.C.1 Membranes lipoprotein	2.2	9.0	8.8E-22	2.2E-21	D1	
dmsC	putative dimethyl sulfoxide reductase subunit C	1.C.5 Sulphur Metabolism	2.2	8.3	5.9E-20	1.4E-19	D1	
STY3461	ATP-dependent RNA helicase (dead-box protein)	3.A.9 RNA synthesis, modification	2.2	7.4	3.3E-17	7.1E-17	D1	
nikR	nickel responsive regulator	2. Broad regulatory function	2.2	8.2	6.7E-20	1.6E-19	D1	
STY3857	pseudogene	5.H Hypothetical protein	2.2	9.4	2.9E-22	7.3E-22	D1	
STY4171	dipeptide transport ATP-binding protein DppD	4.A.6 Transport Other	2.2	9.2	5.6E-22	1.4E-21	D1	
yheS	probable ABC transporter ATP-binding protein	4.A Transport/binding proteins	2.2	10.0	7.8E-23	2.0E-22	D1	
STY4909	conserved hypothetical protein	5.H Hypothetical protein	2.2	9.2	1.4E-21	3.3E-21	D1	

STY3888	hypothetical 20.8 kDa protein in rbsr-rrsc intergenic region	2. Broad regulatory function	2.2	9.7	2.2E-22	5.5E-22	D1	
STY1830	putative outer membrane protein	3.C Cell envelope	2.2	9.0	4.5E-21	1.1E-20	D1	
STY2854	chorismate mutase-P/prephenate dehydratase	1.D.4 Aromatic Amino Acid	2.2	10.0	1.6E-22	4.1E-22	D1	
sscB	putative pathogenicity island protein	4.1 Pathogenesis	2.2	8.7	2.3E-20	5.3E-20	D1	
STY4628	probable capsid portal protein	4.1 Pathogenesis	2.2	9.7	3.7E-22	9.2E-22	D1	
STY0485	cytochrome o ubiquinol oxidase subunit II	1.B.7.a Aerobic Respiration	2.2	9.0	5.8E-21	1.4E-20	D1	
STY1551	putative regulatory protein	2. Broad regulatory function	2.1	8.7	2.2E-20	5.3E-20	D1	
STY3418	conserved hypothetical protein	5.H Hypothetical protein	2.1	8.8	3.1E-20	7.4E-20	D1	
STY0119	L-arabinose isomerase	1.A.1 Degradation of carbohydrate	2.1	8.1	1.4E-18	3.1E-18	D1	
stbE	fimbrial chaperone protein	3.C.3 Surface structure	2.1	8.8	3.3E-20	7.9E-20	D1	
STY4086	2-amino-3-ketobutyrate coenzyme A ligase	1.C Central Intermediary metabolism	2.1	9.2	6.0E-21	1.4E-20	D1	
STY3848	conserved hypothetical protein	5.H Hypothetical protein	2.1	9.1	9.9E-21	2.4E-20	D1	
STY4192	conserved hypothetical protein	5.H Hypothetical protein	2.1	8.8	3.4E-20	8.0E-20	D1	
menD	menaquinone biosynthesis protein	1.G.11 Menaquinone	2.1	8.9	3.4E-20	8.0E-20	D1	
STY3112	1,2-propanediol oxidoreductase (lactaldehyde reductase)	1.A.1 Degradation of carbohydrate	2.1	8.2	1.3E-18	3.0E-18	D1	
STY3666	hypothetical protein	5.I Unknown	2.1	8.0	3.1E-18	6.9E-18	D1	
pilQ	nucleotide-binding protein	4.A Transport/binding proteins	2.1	9.8	2.3E-21	5.6E-21	D1	
STY0484	cytochrome o ubiquinol oxidase subunit I	1.B.7.a Aerobic Respiration	2.1	8.1	1.9E-18	4.1E-18	D1	D8
arcC	carbamate kinase	1.A.1 Degradation of carbohydrate	2.1	8.0	4.5E-18	9.8E-18	D1	
STY4191	2-dehydro-3-deoxygluconokinase	1.A.1 Degradation of carbohydrate	2.1	8.5	3.1E-19	7.0E-19	D1	
STY1565	putative dimethyl sulphoxide reductase subunit	1.A.1 Degradation of carbohydrate	2.1	9.0	4.4E-20	1.0E-19	D1	D8
STY1104	pseudogene	3.C.1 Membranes lipoprotein	2.1	8.1	4.9E-18	1.1E-17	D1	
STY4046	putative purine permease	4.A.6 Transport Other	2.1	9.7	5.7E-21	1.4E-20	D1	
STY0166	prepilin peptidase dependent protein D precursor	4.A Transport/binding proteins	2.1	7.1	6.0E-15	1.2E-14	D1	
STY1661	conserved hypothetical protein	5.H Hypothetical protein	2.1	7.3	1.7E-15	3.4E-15	D1	
mrn	ribonuclease R (RNase R)	3.B.1 Degradation of RNA	2.1	10.1	2.9E-21	7.1E-21	D1	
STY4012	pseudogene	2. Broad regulatory function	2.1	10.7	8.3E-22	2.1E-21	D1	
STY1433	conserved hypothetical protein	5.H Hypothetical protein	2.1	9.0	1.1E-19	2.5E-19	D1	
STY3155	galactose operon repressor	1.A.1 Degradation of carbohydrate	2.1	9.6	2.1E-20	5.0E-20	D1	D8
STY3771	pseudogene	1.C.5 Sulphur Metabolism	2.1	11.0	1.0E-21	2.5E-21	D1	D8
ytfH	conserved hypothetical protein	5.H Hypothetical protein	2.1	8.2	4.6E-18	1.0E-17	D1	
STY4213	universal stress protein B	5.I Unknown	2.1	8.1	1.4E-17	2.9E-17	D1	D8
STY3808	periplasmic sulphate binding protein	1.C.5 Sulphur Metabolism	2.1	10.0	1.2E-20	2.8E-20	D1	
STY1311	conserved hypothetical protein	5.H Hypothetical protein	2.1	8.2	8.2E-18	1.8E-17	D1	
STY1312	cardiolipin synthetase	3.A.11 Phospholipids	2.1	9.7	2.7E-20	6.4E-20	D1	D8
STY2491	ADA regulatory protein	3.A.7 DNA replication, repair, restriction, modification	2.0	6.7	4.2E-13	7.9E-13	D1	

yjfl	putative membrane protein	3.C.1 Membranes lipoprotein	2.0	8.8	6.9E-19	1.6E-18	D1	D8
STY4782	putative membrane protein	3.C.1 Membranes lipoprotein	2.0	8.3	5.2E-18	1.1E-17	D1	D8
STY0790	conserved hypothetical protein	5.H Hypothetical protein	2.0	7.0	3.3E-14	6.4E-14	D1	
STY1412	conserved hypothetical protein	5.H Hypothetical protein	2.0	8.8	5.1E-19	1.2E-18	D1	
fimW	fimbriae w protein	3.C.3 Surface structure	2.0	7.2	9.3E-15	1.8E-14	D1	
STY3693	conserved hypothetical protein	5.I Unknown	2.0	10.0	2.7E-20	6.5E-20	D1	
STY0328	hypothetical protein	4.I Pathogenesis	2.0	8.3	8.9E-18	1.9E-17	D1	
waaQ	lipopolysaccharide core biosynthesis protein	3.C.2 Surface polysacchar	2.0	6.2	2.9E-11	5.1E-11	D1	M3
STY2825	DNA repair protein RecO	3.A.7 DNA replication, repair, restriction, modification	2.0	8.5	6.7E-18	1.5E-17	D1	D8
yjfY	conserved hypothetical protein	5.H Hypothetical protein	2.0	8.4	1.1E-17	2.4E-17	D1	
STY4429	chorismate lyase	1.G.11 Menaquinone	2.0	9.6	1.4E-19	3.2E-19	D1	D8
STY0556	outer membrane protein	3.C Cell envelope	2.0	9.4	2.9E-19	6.6E-19	D1	
STY2036	putative bacteriophage protein	#N/A	2.0	7.9	2.2E-16	4.6E-16	D1	
STY0085	FixA protein	1.A.1 Degradation of carbohydrate	2.0	9.0	1.2E-18	2.6E-18	D1	D8
STY3914	ATP synthase epsilon subunit	1.A Degradation	14.5	11.4	8.9E-187	1.4E-185	D8	D1M3
tufB	elongation factor Tu	3.A.8 Protein translation and modification	13.7	13.0	7.1E-235	4.7E-233	D8	D1M1M3
STY3800	pseudogene	3.A.11 Phospholipids	13.4	13.4	1.4E-246	2.0E-244	D8	
nuoI	NADH dehydrogenase I chain I	1.B.7.a Aerobic Respiration	13.3	12.6	4.1E-223	1.6E-221	D8	M3
trpS	tryptophanyl-tRNA synthetase	3.A.5 Aminoacyl tRNA synthesis, tRNA modification	12.6	12.5	3.3E-220	1.1E-218	D8	D1M1M3
STY1335	conserved hypothetical protein	5.H Hypothetical protein	12.4	13.9	1.6E-263	1.7E-260	D8	
STY3417	conserved hypothetical protein	5.H Hypothetical protein	12.4	12.5	4.5E-222	1.7E-220	D8	M3
STY3913	ATP synthase beta subunit	1.A Degradation	12.4	11.9	1.5E-203	3.0E-202	D8	
STY4213	universal stress protein B	5.I Unknown	12.4	14.8	6.7E-289	2.8E-285	D8	D1
STY2839	conserved hypothetical protein	5.H Hypothetical protein	12.4	14.3	9.1E-274	1.9E-270	D8	D1
STY0483	cytochrome o ubiquinol oxidase subunit III	1.B.7.a Aerobic Respiration	12.0	13.1	4.4E-238	3.6E-236	D8	
STY4910	peptide chain release factor 3	3.A.8 Protein translation and modification	12.0	14.1	7.6E-268	1.1E-264	D8	
STY2145	tyrosine-specific transport protein	4.A.1 Transport amino acid and amines	11.8	13.1	5.2E-239	4.8E-237	D8	D1M3
STY2836	ATP-dependent RNA helicase SrmB	3.A.9 RNA synthesis, modification	11.8	13.3	5.8E-246	7.6E-244	D8	D1M3
STY1609	hypothetical protein	5.I Unknown	11.8	13.1	1.8E-239	1.7E-237	D8	
STY3453	conserved hypothetical protein	5.H Hypothetical protein	11.8	13.9	2.7E-263	2.3E-260	D8	
STY4842	probable regulatory protein	4.I Pathogenesis	11.8	12.3	7.8E-214	2.1E-212	D8	D1
STY1818	putative nitroreductase	5.I Unknown	11.7	13.4	5.0E-247	7.3E-245	D8	
STY4205	glutathione reductase	1.G.10 Thioredoxin	11.7	13.6	1.3E-254	4.0E-252	D8	
STY2546	NADH dehydrogenase I chain N	1.B.7.a Aerobic Respiration	11.7	12.5	1.4E-220	4.8E-219	D8	
STY1578	putative regulatory protein	2. Broad regulatory function	11.6	12.6	3.7E-225	1.6E-223	D8	
STY2918	conserved hypothetical protein	5.H Hypothetical protein	11.5	13.8	3.6E-259	2.1E-256	D8	

ybaD	pseudogene	5.H Hypothetical protein	11.5	13.6	4.5E-254	1.3E-251	D8	M3
fimI	pseudogene	3.C.3 Surface structure	11.5	13.7	5.4E-256	2.2E-253	D8	
STY0777	succinate dehydrogenase flavoprotein subunit	1.B.3 Tricarboxylic acid cycle	11.4	13.1	6.3E-239	5.7E-237	D8	
fliA	RNA polymerase sigma transcription factor for flagellar operon	3.C.3 Surface structure	11.4	13.6	1.3E-254	4.0E-252	D8	
STY2202	putative membrane protein	3.C.1 Membranes lipoprotein	11.4	13.5	2.5E-249	4.2E-247	D8	
glpR	glycerol-3-phosphate regulon repressor	1.B.7.b Anaerobic Respiration	11.3	13.1	3.0E-238	2.5E-236	D8	M3
STY4110	mannitol-1-phosphate dehydrogenase	1.A.1 Degradation of carbohydrate	11.3	12.5	5.4E-222	2.0E-220	D8	
garR	2-hydroxy-3-oxopropionate reductase	1.A.1 Degradation of carbohydrate	11.3	13.7	4.3E-256	2.0E-253	D8	
STY3263	conserved hypothetical protein	5.H Hypothetical protein	11.3	13.7	7.6E-256	2.9E-253	D8	
yafE	putative methyltransferase	5.I Unknown	11.2	13.3	7.8E-244	9.6E-242	D8	
spaN	surface presentation of antigens protein (associated with type III secretion and virulence)	4.I Pathogenesis	11.2	12.9	8.7E-232	5.4E-230	D8	
STY4488	conserved hypothetical protein	5.H Hypothetical protein	11.1	13.6	5.3E-252	1.2E-249	D8	
STY1241	conserved hypothetical protein	5.H Hypothetical protein	11.1	13.4	6.1E-249	9.8E-247	D8	
STY3687	putative phage baseplate assembly protein	5.A Laterally acquired elements	11.1	13.8	2.1E-252	5.1E-250	D8	
STY2678	sulphate transport ATP-binding protein CysA	4.A.5 Transport Anions	11.1	11.6	3.6E-192	6.0E-191	D8	
STY2500	putative MR-MLE-family protein	5.I Unknown	11.1	14.4	1.2E-257	6.5E-255	D8	
appC	cytochrome bd-II oxidase subunit I (pseudogene)	1.B.7.c Electron Transport	11.1	13.8	3.0E-253	7.9E-251	D8	
STY3199	thiol:disulfide interchange protein	3.A.8 Protein translation and modification	11.1	14.1	3.6E-255	1.3E-252	D8	D1
torC	pseudogene	1.B.7.b Anaerobic Respiration	11.1	14.8	3.8E-260	2.7E-257	D8	
ybil	hypothetical Zinc-finger containing protein	5.I Unknown	11.1	12.8	1.1E-229	5.6E-228	D8	
STY2725	phosphoribosylaminoimidazole-succinocarboxamide synthase	1.F.1 Purine ribonucleotide biosynthesis	11.1	13.2	2.2E-242	2.5E-240	D8	
STY0637	hypothetical membrane protein p43	3.C.1 Membranes lipoprotein	11.1	13.9	2.1E-251	4.6E-249	D8	
STY3535	tartrate dehydratase	1.B.8 Fermentation	11.0	13.8	2.5E-250	5.0E-248	D8	
STY4911	Putative periplasmic protein	5.F Adaptions and atypical conditions	11.0	14.0	4.2E-251	8.8E-249	D8	
STY1859	conserved hypothetical protein	5.H Hypothetical protein	10.9	13.0	6.4E-235	4.3E-233	D8	
ycaM	probable transport protein	4.A Transport/binding proteins	10.9	13.8	2.0E-246	2.6E-244	D8	
STY2613	pseudogene	5.H Hypothetical protein	10.9	13.7	1.1E-245	1.4E-243	D8	D1
STY0721	N-acetylglucosamine-6-phosphate deacetylase	1.C.4 Amino sugars	10.9	11.5	3.0E-189	4.9E-188	D8	
oadG	oxaloacetate decarboxylase gamma chain	4.A.2 Transport Cations	10.8	13.9	1.1E-247	1.7E-245	D8	
STY1268	hypothetical protein	5.I Unknown	10.8	14.2	1.8E-249	3.2E-247	D8	
STY2033	putative bacteriophage protein	5.A Laterally acquired elements	10.8	12.6	1.9E-223	7.6E-222	D8	
smg	conserved hypothetical protein	5.H Hypothetical protein	10.8	14.3	3.1E-250	5.9E-248	D8	
slyX	conserved hypothetical protein	5.H Hypothetical protein	10.8	12.8	1.0E-228	5.1E-227	D8	
STY1331	putative pseudouridine synthase	3.A.3 Ribosomal maturation and modification	10.8	13.2	3.2E-242	3.5E-240	D8	

STY1565	putative dimethyl sulphoxide reductase subunit	1.A.1 Degradation of carbohydrate	10.8	14.2	4.6E-248	7.1E-246	D8	D1
STY3875	Two-component system sensory histidine kinase	1.D.1 Glutamate Family	10.8	12.7	1.1E-226	4.9E-225	D8	M3
STY2829	GTP-binding protein LepA	3.A.8 Protein translation and modification	10.8	12.8	4.3E-230	2.4E-228	D8	D1
STY1501	putative secreted protein	3.C.1 Membranes lipoprotein	10.7	12.8	7.5E-231	4.4E-229	D8	D1
STY3819	possible membrane transport protein	4.A Transport/binding proteins	10.7	13.6	4.4E-242	4.7E-240	D8	
STY3262	conserved hypothetical protein	5.H Hypothetical protein	10.7	14.8	4.9E-250	9.0E-248	D8	
STY1539	putative membrane protein	3.C.1 Membranes lipoprotein	10.7	13.6	8.8E-241	9.0E-239	D8	M3
STY0186	putative transcriptional regulator	2. Broad regulatory function	10.6	13.6	1.8E-240	1.8E-238	D8	D1
ygaU	pseudogene	5.H Hypothetical protein	10.6	12.5	9.5E-222	3.5E-220	D8	
STY0553	hypothetical ABC transporter ATP-binding protein	4.A Transport/binding proteins	10.6	13.4	2.5E-238	2.2E-236	D8	
hslT	heat shock protein A	5.F Adaptions and atypical conditions	10.6	14.1	6.0E-243	7.2E-241	D8	
STY1129	putative exported protein	3.C.1 Membranes lipoprotein	10.5	12.3	1.7E-215	4.7E-214	D8	
STY2746	hypothetical protein	5.I Unknown	10.5	13.4	4.1E-236	3.2E-234	D8	
STY1222	flagellar hook-associated protein 1	3.C.3 Surface structure	10.5	13.5	7.2E-236	5.3E-234	D8	
STY2675	putative amidotransferase	5.I Unknown	10.5	12.3	5.5E-216	1.6E-214	D8	M3
envE	putative lipoprotein	3.C.1 Membranes lipoprotein	10.5	11.9	2.1E-202	4.2E-201	D8	
STY2770	conserved hypothetical protein	5.H Hypothetical protein	10.5	13.6	2.3E-236	1.8E-234	D8	
rrmA	rRNA guanine-N1-methyltransferase	3.A.9 RNA synthesis, modification	10.5	12.8	9.2E-230	4.9E-228	D8	
STY1173	conserved hypothetical protein	5.H Hypothetical protein	10.4	13.1	9.4E-233	5.9E-231	D8	
STY2565	conserved hypothetical protein	5.H Hypothetical protein	10.4	12.8	5.2E-230	2.8E-228	D8	
spaQ	secretory protein (associated with virulence)	4.I Pathogenesis	10.4	10.6	6.9E-163	8.3E-162	D8	
STY2443	sugar efflux transporter	4.A Transport/binding proteins	10.4	13.5	4.4E-235	3.0E-233	D8	M3
STY2437	putative membrane protein	3.C.1 Membranes lipoprotein	10.4	13.4	5.9E-234	3.8E-232	D8	
STY1945	septum site determining protein	4.C Cell division	10.4	13.7	1.6E-235	1.1E-233	D8	
STY2389	putative two-component system sensor kinase	2. Broad regulatory function	10.4	14.0	2.9E-238	2.4E-236	D8	
STY2779	putative thiosulfate sulfurtransferase	1.D.4 Aromatic Amino Acid	10.4	12.6	1.5E-223	5.9E-222	D8	
STY3376	glycogen synthesis protein GlgS	3.A.10 Polysaccharides (Cytoplasmic)	10.4	11.3	1.0E-183	1.5E-182	D8	
stbC	outer membrane fimbrial usher protein	3.C.3 Surface structure	10.4	14.6	2.8E-242	3.2E-240	D8	
STY3847	conserved hypothetical protein	5.H Hypothetical protein	10.4	13.8	4.4E-236	3.4E-234	D8	
STY2472	nitrate/nitrite response regulator protein NarP	1.B.7.b Anaerobic Respiration	10.4	11.9	6.9E-204	1.4E-202	D8	
STY2916	conserved hypothetical protein	5.H Hypothetical protein	10.4	12.8	1.6E-229	8.2E-228	D8	
STY0753	conserved hypothetical protein	5.H Hypothetical protein	10.3	12.9	1.3E-229	6.6E-228	D8	
mdlA	putative ABC transporter ATP-binding membrane protein	4.A Transport/binding proteins	10.3	13.9	1.2E-235	9.0E-234	D8	
STY4928	conserved hypothetical ABC transporter	4.A Transport/binding proteins	10.3	14.6	8.6E-241	9.0E-239	D8	
STY2113	conserved hypothetical protein	5.H Hypothetical protein	10.3	14.1	2.3E-237	1.8E-235	D8	
STY4466	putative xanthine/uracil permeases family protein	4.A Transport/binding proteins	10.3	14.3	1.6E-238	1.5E-236	D8	

STY2082	DNA polymerase III, theta subunit	#N/A	10.3	11.5	2.9E-191	4.8E-190	D8	
ygcX	probable glucarate dehydratase 1	1.A.1 Degradation of carbohydrate	10.3	14.0	5.5E-236	4.1E-234	D8	
STY4422	putative membrane protein	3.C.1 Membranes lipoprotein	10.3	13.4	6.4E-231	3.8E-229	D8	
STY0220	ferrichrome-binding periplasmic protein precursor	4.A.6 Transport Other	10.3	13.1	6.2E-229	3.1E-227	D8	
STY4668	conserved hypothetical protein	4.1 Pathogenesis	10.3	14.8	2.0E-240	2.0E-238	D8	
bcr	bicyclomycin resistance protein	5.D Drug/Analogue sensitivity	10.2	13.6	4.1E-231	2.5E-229	D8	
STY3465	tRNA pseudouridine 55 synthase (psi55 synthase) (p35 protein)	3.A.5 Aminoacyl tRNA synthesis, tRNA modification	10.2	13.6	5.5E-231	3.3E-229	D8	
befA	fimbrial subunit	3.C.3 Surface structure	10.2	13.2	2.2E-228	1.0E-226	D8	D1
STY2928	conserved hypothetical protein	5.H Hypothetical protein	10.2	13.1	7.5E-227	3.5E-225	D8	
STY4439	conserved hypothetical protein	5.H Hypothetical protein	10.2	13.5	5.2E-229	2.6E-227	D8	
STY3312	hypothetical protein	5.1 Unknown	10.2	11.8	2.8E-200	5.4E-199	D8	
btuE	putative glutathione peroxidase/vitamin B12 transport periplasmic protein BtuE	4.A.6 Transport Other	10.2	12.0	7.0E-207	1.6E-205	D8	
ybjZ	conserved hypothetical ABC transporter	4.A Transport/binding proteins	10.2	14.4	2.7E-235	1.9E-233	D8	
STY2005	conserved hypothetical protein	5.H Hypothetical protein	10.2	13.4	2.6E-228	1.2E-226	D8	
STY3115	L-fucose permease	4.A.3 Transport Carbohydrates, organic acids and alcohols	10.1	13.7	4.1E-230	2.3E-228	D8	
STY2414	vancomycin resistance protein	5.D Drug/Analogue sensitivity	10.1	13.6	1.9E-228	9.4E-227	D8	
stbB	fimbrial chaperone protein	3.C.3 Surface structure	10.1	12.4	6.1E-217	1.8E-215	D8	
STY3834	hypothetical protein	5.1 Unknown	10.1	14.4	1.1E-233	6.8E-232	D8	
STY3463	polynucleotide phosphorylase	3.A.9 RNA synthesis, modification	10.1	14.6	1.1E-234	7.2E-233	D8	D1M1M3
STY4206	conserved hypothetical protein	5.H Hypothetical protein	10.1	12.9	1.6E-222	6.2E-221	D8	D1
ygbJ	pseudogene	5.1 Unknown	10.1	12.6	4.8E-221	1.6E-219	D8	
STY4139	putative membrane protein	3.C.1 Membranes lipoprotein	10.1	14.0	4.7E-230	2.6E-228	D8	
STY2548	NADH dehydrogenase I chain L	1.B.7.a Aerobic Respiration	10.1	10.3	4.5E-154	5.1E-153	D8	
STY2734	putative membrane protein	3.C.1 Membranes lipoprotein	10.1	13.4	8.0E-226	3.5E-224	D8	
STY0881	conserved hypothetical protein	5.H Hypothetical protein	10.0	12.4	5.3E-218	1.6E-216	D8	
STY2809	putative transcriptional regulator	2. Broad regulatory function	10.0	13.3	1.1E-224	4.6E-223	D8	
STY3108	conserved hypothetical protein	5.H Hypothetical protein	10.0	14.1	1.1E-230	6.4E-229	D8	
STY1571	putative ABC transporter periplasmic binding protein	4.A Transport/binding proteins	10.0	12.4	1.2E-218	3.6E-217	D8	
STY1650	conserved hypothetical protein	5.H Hypothetical protein	10.0	13.5	1.8E-226	8.3E-225	D8	
STY3568	putative exported protein	3.C.1 Membranes lipoprotein	10.0	14.0	1.5E-229	7.5E-228	D8	
STY1587	putative membrane protein	3.C.1 Membranes lipoprotein	10.0	12.9	4.8E-221	1.6E-219	D8	
steF	fimbrial subunit	3.C.3 Surface structure	10.0	13.7	1.1E-226	5.2E-225	D8	
STY1568	putative dimethyl sulphoxide reductase subunit	1.A.1 Degradation of carbohydrate	10.0	12.0	2.2E-204	4.7E-203	D8	
STY3313	possible ABC-transport protein, periplasmic-binding component	4.A Transport/binding proteins	10.0	12.7	1.6E-218	4.8E-217	D8	

pipA	conserved hypothetical protein	4.1 Pathogenesis	10.0	13.6	2.0E-225	8.8E-224	D8	
STY3803	hypothetical protein	5.1 Unknown	10.0	13.5	5.1E-224	2.1E-222	D8	
STY0010	conserved hypothetical protein	5.H Hypothetical protein	9.9	13.7	6.3E-225	2.7E-223	D8	
STY4201	gntR family regulatory protein	2. Broad regulatory function	9.9	12.7	2.1E-216	6.2E-215	D8	
ybaV	putative exported protein	3.C.1 Membranes lipoprotein	9.9	12.5	6.7E-216	1.9E-214	D8	
STY0124	putative ABC transporter integral membrane protein	4.A Transport/binding proteins	9.9	13.4	1.4E-222	5.5E-221	D8	
STY2398	pseudogene	3.C.4 Murein sacculus and peptidoglycan	9.9	13.1	2.7E-220	8.6E-219	D8	
STY0867	glutamine transport system permease protein GlnP	4.A.1 Transport amino acid and amines	9.9	13.3	6.8E-221	2.3E-219	D8	
STY1252	putative secreted protein	3.C.1 Membranes lipoprotein	9.9	12.0	1.2E-206	2.7E-205	D8	
STY4154	putative DNA-binding protein	5.1 Unknown	9.9	13.9	5.8E-226	2.6E-224	D8	
STY2159	putative ABC-transport ATP-binding protein	4.A Transport/binding proteins	9.9	13.6	1.4E-223	5.8E-222	D8	
sugE	SugE protein	4.B Chaperones	9.9	13.6	8.7E-223	3.4E-221	D8	
STY2913	GabA permease (4-amino butyrate transport carrier)	4.A.3 Transport Carbohydres, organic acids and alcohols	9.9	13.7	1.6E-223	6.3E-222	D8	
STY4262	putative membrane protein	3.C.1 Membranes lipoprotein	9.9	13.9	3.5E-225	1.5E-223	D8	
ttrA	tetrathionate reductase subunit A	4.1 Pathogenesis	9.9	13.3	1.5E-220	5.1E-219	D8	
STY3623	uroporphyrinogen III methylase	1.G.12 Heme and porphyrin	9.8	13.2	9.0E-220	2.8E-218	D8	
STY3125	conserved hypothetical protein	5.H Hypothetical protein	9.8	13.5	2.2E-221	8.0E-220	D8	
STY3934	conserved hypothetical protein	5.H Hypothetical protein	9.8	13.5	2.7E-221	9.4E-220	D8	
STY3951	Two-component sensor protein histidine protein kinase.	1.B.7.b Anaerobic Respiration	9.8	14.9	5.6E-230	3.0E-228	D8	
STY0620	oxygen-insensitive NAD(P)H nitroreductase	5.D Drug/Analogue sensitivity	9.8	13.5	3.7E-221	1.3E-219	D8	
STY1549	dipeptidyl carboxypeptidase II	3.B.3 Degradation of proteins, peptides, glycoproteins	9.8	13.5	2.0E-221	7.2E-220	D8	
STY1680	putative HlyD-family protein	5.1 Unknown	9.8	13.1	3.4E-217	1.0E-215	D8	
yjfC	conserved hypothetical protein	5.H Hypothetical protein	9.8	12.7	4.2E-214	1.1E-212	D8	
fliB	pseudogene	#N/A	9.8	13.8	1.8E-222	6.7E-221	D8	
STY0927	putative N-acetylmutamoyl-L-alanine amidase	3.C.4 Murein sacculus and peptidoglycan	9.8	12.2	1.9E-211	4.8E-210	D8	
dsbG	thiol:disulfide interchange protein DsbG precursor	1.C Central Intermediary metabolism	9.8	12.9	2.8E-215	7.8E-214	D8	
pocR	pdu/cob regulatory protein PocR	2. Broad regulatory function	9.7	13.1	5.7E-216	1.6E-214	D8	
STY3989	putative carbohydrate kinase	1.A.1 Degradation of carbohydrate	9.7	14.2	8.7E-224	3.6E-222	D8	
STY4429	chorismate lyase	1.G.11 Menaquinone	9.7	13.8	1.2E-221	4.5E-220	D8	D1
STY4407	peptidase E	3.B.3 Degradation of proteins, peptides, glycoproteins	9.7	13.6	8.1E-220	2.6E-218	D8	
STY3751	acetylglutamate kinase	1.D.1 Glutamate Family	9.7	13.2	3.0E-216	8.6E-215	D8	
STY0165	protein transport protein HofB	4.A.6 Transport Other	9.7	13.1	7.8E-216	2.2E-214	D8	
STY1796	conserved hypothetical protein	5.H Hypothetical protein	9.7	12.7	2.4E-212	6.3E-211	D8	
ybhL	pseudogene	3.C.1 Membranes lipoprotein	9.7	14.1	2.1E-222	7.9E-221	D8	
STY3580	flavin reductase	1.B.7.c Electron Transport	9.7	12.3	1.5E-208	3.6E-207	D8	M3
STY1160	sodium/proline symporter (proline permease)	4.A.1 Transport amino acid and amines	9.7	13.9	1.6E-220	5.2E-219	D8	

STY3694	hypothetical protein	5.I Unknown	9.6	14.1	4.0E-221	1.4E-219	D8	
STY3810	putative transmembrane efflux protein	4.A Transport/binding proteins	9.6	14.0	1.6E-220	5.2E-219	D8	
cheM	methyl-accepting chemotaxis protein II	4.D Chemotaxis and mobility	9.6	14.1	5.3E-221	1.8E-219	D8	
STY4636	DNA adenine methylase	4.I Pathogenesis	9.6	13.5	4.5E-217	1.3E-215	D8	
STY2932	ribonucleoside-diphosphate reductase 2 alpha chain	1.F.5 Miscellaneous	9.6	14.0	1.9E-220	6.0E-219	D8	
fliT	flagellar protein FliT	3.C.3 Surface structure	9.6	11.3	2.0E-183	2.9E-182	D8	
hpaG	4-hydroxyphenylacetate degradation bifunctional isomerase/decarboxylase [includes: 2-hydroxyhepta-2,4-diene-1,7-dioate isomerase]	1.A.1 Degradation of carbohydrate	9.6	12.4	1.3E-208	3.2E-207	D8	
hslS	heat shock protein B	5.F Adaptions and atypical conditions	9.6	13.7	1.3E-217	3.9E-216	D8	
ppiA	peptidyl-prolyl cis-trans isomerase	3.A.8 Protein translation and modification	9.6	13.2	3.2E-214	8.6E-213	D8	
STY1289	respiratory nitrate reductase 1 beta chain	1.B.7.b Anaerobic Respiration	9.6	13.3	1.4E-214	3.7E-213	D8	
pgtB	phosphoglycerate transport system sensor protein PgtB	4.A Transport/binding proteins	9.6	13.7	6.9E-217	2.0E-215	D8	
STY0538	fosmidomycin resistance protein	4.A.6 Transport Other	9.5	13.0	1.3E-211	3.3E-210	D8	
trkH	trk system potassium uptake protein	1.C.5 Sulphur Metabolism	9.5	10.9	1.3E-171	1.6E-170	D8	D1M3
cbiA	cobyrinic acid A,C-diamide synthase	1.G.13 Cobalamin	9.5	12.7	8.0E-209	1.9E-207	D8	
STY3636	lipopolysaccharide biosynthesis protein	3.C.2 Surface polysacchar	9.5	13.7	5.6E-216	1.6E-214	D8	
STY2179	flagellar assembly protein FliH	3.C.3 Surface structure	9.5	12.1	2.1E-203	4.4E-202	D8	
STY4447	conserved hypothetical protein	5.H Hypothetical protein	9.5	11.5	1.4E-191	2.4E-190	D8	
gtrA	bactoprenol-linked glucose translocase	3.C.2 Surface polysacchar	9.5	13.0	5.4E-210	1.3E-208	D8	M1
tetE	putative two-component system sensor kinase	2. Broad regulatory function	9.5	13.1	1.1E-210	2.7E-209	D8	
nirC	putative nitrite transporter	4.A.5 Transport Anions	9.5	14.0	2.4E-216	6.9E-215	D8	
STY4820	pseudogene	5.I Unknown	9.5	13.4	2.1E-212	5.5E-211	D8	
STY1384	periplasmic murein peptide-binding protein MppA	4.A.6 Transport Other	9.5	13.5	7.5E-213	2.0E-211	D8	
STY2646	putative decarboxylase	5.I Unknown	9.4	14.4	6.2E-218	1.9E-216	D8	
STY2004	putative hydrolase	5.I Unknown	9.4	13.5	9.4E-212	2.4E-210	D8	
STY3709	phosphoribosylaminoimidazolecarboxamide formyltransferase and IMP cyclohydrolase (bifunctional enzyme)	1.F.1 Purine ribonucleotide biosynthesis	9.4	13.8	7.2E-214	1.9E-212	D8	
mntH	manganese transport protein MntH	4.A Transport/binding proteins	9.4	12.8	7.7E-207	1.8E-205	D8	
nlpC	putative lipoprotein	3.C.1 Membranes lipoprotein	9.4	13.3	3.3E-210	8.1E-209	D8	
STY4901	conserved hypothetical regulatory protein	2. Broad regulatory function	9.4	13.5	7.6E-212	2.0E-210	D8	
STY1615	conserved hypothetical protein	5.H Hypothetical protein	9.4	13.3	5.4E-210	1.3E-208	D8	
STY2211	AMP nucleosidase	1.F.4 Salvage of nucleosides and nucleotides	9.4	13.5	3.0E-211	7.5E-210	D8	
STY2650	nucleoside permease NupC	4.A.6 Transport Other	9.4	12.9	1.4E-206	3.3E-205	D8	D1M3
STY3481	putative membrane protein	3.C.1 Membranes lipoprotein	9.4	13.4	1.6E-210	4.1E-209	D8	
STY3133	protease III precursor (pitirylisin)	3.B.3 Degradation of proteins, peptides, glycoproteins	9.4	14.0	7.9E-214	2.1E-212	D8	



STY0865	putative membrane protein	3.C.1 Membranes lipoprotein	9.3	12.9	4.0E-206	8.9E-205	D8	D1
citC	citrate-sodium symporter	4.A.3 Transport Carbohydes, organic acids and alcohols	9.3	13.2	2.9E-208	6.7E-207	D8	
STY0306	putative membrane protein	4.I Pathogenesis	9.3	12.9	8.6E-206	1.9E-204	D8	
STY0273	putative ABC transporter permease protein	4.A Transport/binding proteins	9.3	12.9	1.6E-205	3.4E-204	D8	
STY1660	putative oxidoreductase	5.I Unknown	9.3	12.6	1.5E-203	3.1E-202	D8	
STY1835	conserved hypothetical protein	5.H Hypothetical protein	9.3	12.2	3.7E-200	7.1E-199	D8	
STY0612	probable pyridine nucleotide-disulfide oxidoreductase	5.I Unknown	9.3	13.0	1.6E-205	3.4E-204	D8	
STY2396	periplasmic beta-glucosidase precursor	1.A.1 Degradation of carbohydrate	9.3	13.6	9.1E-210	2.2E-208	D8	
yheR	putative oxidoreductase	5.I Unknown	9.3	12.4	6.9E-201	1.3E-199	D8	
STY4297	putative membrane protein	3.C.1 Membranes lipoprotein	9.3	13.6	4.7E-209	1.1E-207	D8	
gltP	proton glutamate symport protein	4.A.1 Transport amino acid and amines	9.3	13.1	1.7E-205	3.7E-204	D8	
STY2966	putative exported protein	3.C.1 Membranes lipoprotein	9.2	12.4	1.7E-200	3.2E-199	D8	
tdcE	probable formate acetyltransferase	1.B.7.b Anaerobic Respiration	9.2	13.7	3.4E-209	8.1E-208	D8	
STY0164	protein transport protein HofC	4.A.6 Transport Other	9.2	12.0	7.6E-196	1.3E-194	D8	
hyaB2	pseudogene	1.B.7.a Aerobic Respiration	9.2	12.3	3.7E-199	6.8E-198	D8	
STY4933	right origin-binding protein	3.A.7 DNA replication, repair, restriction, modification	9.2	13.2	4.6E-205	1.0E-203	D8	
STY2530	conserved hypothetical protein	5.H Hypothetical protein	9.2	12.6	4.2E-201	8.1E-200	D8	
STY4593	hypothetical protein	4.I Pathogenesis	9.2	12.3	4.4E-198	8.1E-197	D8	
STY2428	putative esterase	5.I Unknown	9.2	11.7	2.1E-193	3.5E-192	D8	
STY0194	hypothetical ABC transporter ATP-binding protein	4.A Transport/binding proteins	9.2	12.7	2.1E-201	4.2E-200	D8	D1
STY0215	penicillin-binding protein 1b; peptidoglycan synthetase	3.C.4 Murein sacculus and peptidoglycan	9.2	10.1	4.9E-148	5.2E-147	D8	
STY3617	conserved hypothetical protein	5.H Hypothetical protein	9.2	13.3	5.5E-205	1.2E-203	D8	
STY4406	putative membrane protein	3.C.1 Membranes lipoprotein	9.2	13.6	1.3E-206	3.0E-205	D8	
tviA	Vi polysaccharide biosynthesis protein	4.I Pathogenesis	9.2	13.6	3.5E-206	7.9E-205	D8	
ygcY	probable glucarate dehydratase 2	1.A.1 Degradation of carbohydrate	9.1	13.3	7.7E-205	1.7E-203	D8	
STY1694	putative integral membrane transport protein	4.A Transport/binding proteins	9.1	12.2	5.9E-196	1.1E-194	D8	
STY3802	putative GntR-family regulatory protein	2. Broad regulatory function	9.1	13.4	1.3E-204	2.8E-203	D8	
glgX	glycogen operon protein	3.B.4 Degradations of polysaccharides	9.1	13.9	1.1E-207	2.5E-206	D8	
STY3902	regulatory protein	1.D.2 Aspartate Family	9.1	12.1	6.4E-195	1.1E-193	D8	
STY3947	conserved hypothetical protein	5.H Hypothetical protein	9.1	13.4	3.1E-204	6.6E-203	D8	
STY2598	DedA protein (dsg-1 protein)	5.I Unknown	9.1	12.7	2.0E-199	3.8E-198	D8	
STY4200	cytoplasmic trehalase	1.A.1 Degradation of carbohydrate	9.1	13.4	5.8E-204	1.2E-202	D8	
STY2989	hypothetical protein found within S. typhi pathogenicity island 1	4.I Pathogenesis	9.1	14.0	2.6E-207	6.1E-206	D8	
STY0621	conserved hypothetical protein	5.H Hypothetical protein	9.1	12.3	9.7E-196	1.7E-194	D8	
STY4129	conserved hypothetical protein	5.H Hypothetical protein	9.1	12.1	2.3E-194	3.9E-193	D8	
STY2429	putative DNA-binding protein	5.I Unknown	9.1	10.8	3.1E-168	4.0E-167	D8	M3

ybjF	hypothetical RNA methyltransferase	5.I Unknown	9.1	13.0	6.6E-200	1.2E-198	D8	
STY1750	putative aminotransferase	1.C Central Intermediary metabolism	9.1	12.3	5.0E-195	8.8E-194	D8	
STY2732	pseudogene	5.H Hypothetical protein	9.0	13.3	4.8E-202	9.6E-201	D8	
nrdD	anaerobic ribonucleoside-triphosphate reductase	1.F.3 2'-deoxyribonucleotide metabolism	9.0	13.3	7.0E-202	1.4E-200	D8	
STY3590	putative membrane protein	3.C.1 Membranes lipoprotein	9.0	12.8	1.2E-198	2.2E-197	D8	D1
STY1362	pseudogene	4.I Pathogenesis	9.0	8.0	1.5E-79	1.2E-78	D8	
STY2940	Multi-drug resistance protein A	5.D Drug/Analogue sensitivity	9.0	13.3	1.2E-201	2.4E-200	D8	
STY3479	Penicillin-binding protein (D-alanyl-D-alanine carboxypeptidase)	3.C.4 Murein sacculus and peptidoglycan	9.0	13.4	1.4E-201	2.8E-200	D8	
STY3593	putative regulatory protein	2. Broad regulatory function	9.0	12.6	4.0E-196	7.2E-195	D8	
cII	phage regulatory protein	5.A Laterally acquired elements	9.0	11.0	2.6E-176	3.5E-175	D8	M3
STY0014	putative regulatory protein	2. Broad regulatory function	9.0	14.0	8.5E-205	1.8E-203	D8	
STY1504	putative hydrolase	5.I Unknown	9.0	12.3	3.2E-194	5.4E-193	D8	
STY3320	hydrogenase-2 small subunit	1.B.7.b Anaerobic Respiration	9.0	12.7	3.3E-196	5.9E-195	D8	
citF	citrate lyase alpha chain	1.C Central Intermediary metabolism	9.0	12.4	3.5E-194	5.9E-193	D8	
fliZ	FliZ protein	5.I Unknown	9.0	10.6	9.4E-163	1.1E-161	D8	
luxS	autoinducer-2 production protein LuxS	2. Broad regulatory function	9.0	13.7	1.5E-202	3.1E-201	D8	
STY3306	D-mannonate hydrolase	1.A.1 Degradation of carbohydrate	9.0	12.9	2.6E-197	4.8E-196	D8	
STY4020	conserved hypothetical protein	5.H Hypothetical protein	9.0	12.4	1.4E-193	2.3E-192	D8	
STY2873	conserved hypothetical protein	5.H Hypothetical protein	9.0	6.5	9.5E-35	7.2E-34	D8	
STY2933	ribonucleoside-diphosphate reductase 2 beta chain	1.F.5 Miscellaneous	8.9	13.8	2.9E-202	5.9E-201	D8	
menF	isochorismate synthase	1.G.11 Menaquinone	8.9	12.2	2.4E-191	3.9E-190	D8	
STY0680	pseudogene	5.I Unknown	8.9	11.7	7.1E-188	1.1E-186	D8	
relA	GTP pyrophosphokinase	2. Broad regulatory function	8.9	13.6	2.3E-201	4.5E-200	D8	
STY4152	putative exported protein	3.C.1 Membranes lipoprotein	8.9	13.8	1.2E-201	2.3E-200	D8	
ggt	gamma-glutamyltranspeptidase precursor	1.G.10 Thioredoxin	8.9	12.7	8.7E-195	1.5E-193	D8	
STY4168	periplasmic dipeptide transport protein precursor	4.A.6 Transport Other	8.9	13.5	1.6E-199	3.0E-198	D8	
STY2421	galactoside transport system permease protein MglC	4.A.3 Transport Carbohydrates, organic acids and alcohols	8.9	12.0	2.7E-189	4.3E-188	D8	
STY2404	conserved hypothetical protein	5.H Hypothetical protein	8.9	13.0	6.3E-196	1.1E-194	D8	
STY3078		5.H Hypothetical protein	8.9	12.8	1.4E-194	2.4E-193	D8	
dcuR	two-component response regulator	2. Broad regulatory function	8.9	13.8	1.8E-200	3.4E-199	D8	
STY1079	dihydroorotate dehydrogenase	1.F.2 Pyrimidine ribonucle	8.8	13.8	2.9E-200	5.5E-199	D8	
kdgR	probable global regulatory protein	2. Broad regulatory function	8.8	11.7	8.8E-186	1.3E-184	D8	
mdoB	putative phosphoglycerol transferase	5.I Unknown	8.8	14.4	1.4E-201	2.7E-200	D8	
STY3708	possible LysR-family transcriptional regulatory protein	2. Broad regulatory function	8.8	14.2	5.3E-200	1.0E-198	D8	
STY2022	putative exported protein	5.I Unknown	8.8	9.7	6.3E-134	6.3E-133	D8	
mltC	membrane-bound lytic murein transglycosylase C	3.C.4 Murein sacculus and peptidoglycan	8.7	13.1	6.4E-194	1.1E-192	D8	

STY4116	aldehyde dehydrogenase B	1.A.1 Degradation of carbohydrate	8.7	13.3	1.5E-194	2.6E-193	D8	
yhjL	pseudogene	3.C.2 Surface polysacchar	8.7	13.7	1.2E-196	2.2E-195	D8	
STY3980	hypothetical protein	5.I Unknown	8.7	12.4	2.4E-188	3.7E-187	D8	
STY1286	nitrate/nitrite sensor protein NarX	1.B.7.b Anaerobic Respiration	8.7	12.7	7.7E-191	1.3E-189	D8	
STY3534	tartrate dehydratase	1.B.8 Fermentation	8.7	13.6	3.6E-196	6.5E-195	D8	
viaH	pseudogene	3.C.1 Membranes lipoprotein	8.7	14.3	3.0E-198	5.5E-197	D8	
STY4193	putative membrane protein	3.C.1 Membranes lipoprotein	8.7	13.3	5.1E-194	8.6E-193	D8	
STY2577	conserved hypothetical protein	5.H Hypothetical protein	8.7	11.3	2.8E-179	4.0E-178	D8	
STY0294	ClpB-like protein	4.I Pathogenesis	8.7	12.6	2.3E-188	3.6E-187	D8	
sgaT	putative transport protein SgaT	4.A Transport/binding proteins	8.7	12.4	2.2E-186	3.3E-185	D8	
STY2851	ftsH suppressor protein SfhB	3.A.2 Ribosomal protein synthesis, modification	8.6	12.3	9.0E-186	1.4E-184	D8	D1M3
STY0641	isochorismatase	1.G.14 Iron uptake and storage	8.6	10.9	1.2E-172	1.6E-171	D8	
STY4573	hypothetical protein	4.I Pathogenesis	8.6	13.8	3.1E-194	5.3E-193	D8	
STY4570	putative exported protein	4.I Pathogenesis	8.6	13.2	6.5E-191	1.1E-189	D8	
yhjJ	putative zinc-protease precursor	3.B.3 Degradation of proteins, peptides, glycoproteins	8.6	12.8	4.2E-189	6.6E-188	D8	
STY0489	trigger factor	4.C Cell division	8.6	12.7	2.3E-187	3.6E-186	D8	
STY2944	gamma-glutamylcysteine synthetase	1.G.10 Thioredoxin	8.6	10.5	6.3E-161	7.4E-160	D8	M1
STY3332	biopolymer transport ExbB protein	4.A Transport/binding proteins	8.6	11.3	2.0E-177	2.7E-176	D8	
STY1649	outer membrane protein	4.A Transport/binding proteins	8.6	13.0	5.4E-189	8.5E-188	D8	
STY4091	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	5.I Unknown	8.6	13.9	3.5E-193	5.8E-192	D8	
STY1097	helicase IV (75 kD helicase)	3.A.7 DNA replication, repair, restriction, modification	8.5	12.8	1.4E-187	2.2E-186	D8	
rhIB	putative ATP-dependent RNA helicase	3.A.9 RNA synthesis, modification	8.5	12.6	1.0E-185	1.5E-184	D8	
ompX	outer membrane protein x precursor	3.C Cell envelope	8.5	11.8	7.4E-180	1.1E-178	D8	
STY3656	acetolactate synthase large subunit	1.D.6 Pyruvate Family	8.5	13.1	2.6E-188	4.0E-187	D8	
STY0605	putative membrane protein	3.C.1 Membranes lipoprotein	8.5	14.0	1.5E-191	2.5E-190	D8	M1
STY2455	putative ABC-transporter ATP-binding protein	5.H Hypothetical protein	8.5	11.4	3.6E-174	4.8E-173	D8	
STY3337	probable alcohol dehydrogenase	5.I Unknown	8.4	12.7	5.7E-184	8.5E-183	D8	
STY3754	phosphoenolpyruvate carboxylase	1.B.8 Fermentation	8.4	13.8	6.5E-189	1.0E-187	D8	
dpiA	transcriptional regulatory protein dpiA	2. Broad regulatory function	8.4	11.0	4.8E-171	6.2E-170	D8	
STY3783	glycerol uptake facilitator protein	4.A.3 Transport Carbohydrates, organic acids and alcohols	8.4	12.0	5.3E-178	7.4E-177	D8	
STY1566	putative dimethyl sulfoxide reductase subunit	1.A.1 Degradation of carbohydrate	8.4	12.3	4.8E-180	6.9E-179	D8	
STY4851	conserved hypothetical protein	4.I Pathogenesis	8.4	13.2	3.3E-185	5.0E-184	D8	
STY4059	conserved hypothetical protein	5.H Hypothetical protein	8.4	14.1	9.6E-189	1.5E-187	D8	
mtgA	monofunctional biosynthetic peptidoglycan transglycosylase	3.C.4 Murein sacculus and peptidoglycan	8.4	11.7	2.0E-174	2.7E-173	D8	D1
STY2602	Div protein	4.C Cell division	8.3	12.2	1.6E-178	2.3E-177	D8	

STY4821	integrase	#N/A	8.3	13.2	1.7E-184	2.5E-183	D8	
STY3692	hypothetical protein	5.I Unknown	8.3	13.1	7.6E-184	1.1E-182	D8	
STY0362	xanthine-guanine phosphoribosyltransferase	1.F.4 Salvage of nucleosides and nucleotides	8.3	11.5	1.8E-172	2.4E-171	D8	
STY1103	conserved hypothetical protein	5.H Hypothetical protein	8.3	11.3	6.1E-171	7.8E-170	D8	
STY1164	putative membrane transporter	4.A Transport/binding proteins	8.3	11.1	2.5E-168	3.1E-167	D8	
ushA	pseudogene	1.C.3 Sugar Nuclotides	8.3	12.9	1.8E-181	2.6E-180	D8	
corB	putative membrane protein	3.C.1 Membranes lipoprotein	8.3	12.8	1.2E-180	1.8E-179	D8	
STY0631	enterobactin synthetase component F	4.I Pathogenesis	8.3	11.6	2.6E-172	3.4E-171	D8	
STY1534	putative membrane protein	3.C.1 Membranes lipoprotein	8.3	12.5	4.5E-178	6.2E-177	D8	
STY0308	pseudogene	4.I Pathogenesis	8.2	12.8	1.3E-179	1.8E-178	D8	
STY4594	hypothetical protein	4.I Pathogenesis	8.2	11.5	1.5E-170	2.0E-169	D8	D1
yhjA	probable cytochrome c peroxidase	5.I Unknown	8.2	12.9	6.3E-180	9.1E-179	D8	
STY3983	pseudogene	3.C.1 Membranes lipoprotein	8.2	12.4	5.9E-177	8.1E-176	D8	
STY0077	carbamoyl-phosphate synthase large chain	1.F.2 Pyrimidine ribonucle	8.2	12.8	4.9E-179	6.9E-178	D8	
yjeM	putative amino acid permease	4.A Transport/binding proteins	8.2	14.1	6.9E-184	1.0E-182	D8	
STY2791	putative RNA methyltransferase	3.A.9 RNA synthesis, modification	8.2	10.9	2.0E-164	2.5E-163	D8	
hutU	urocanate hydratase	1.A.2 Degradation of amino acids	8.2	11.5	1.1E-168	1.4E-167	D8	
STY4209	oligopeptidase A	3.B.3 Degradation of proteins, peptides, glycoproteins	8.2	12.7	2.3E-177	3.2E-176	D8	
STY2382	conserved hypothetical protein	5.H Hypothetical protein	8.1	14.2	4.5E-183	6.6E-182	D8	
STY4188	conserved hypothetical protein	5.H Hypothetical protein	8.1	13.5	9.9E-181	1.5E-179	D8	
gpsA	glycerol-3-phosphate dehydrogenase	1.B.7.a Aerobic Respiration	8.1	12.0	1.3E-172	1.8E-171	D8	
acT	hypothetical transcriptional activator	2. Broad regulatory function	8.1	11.0	5.4E-164	6.5E-163	D8	
mdfA	Multi-drug translocase MdfA	4.A.6 Transport Other	8.1	12.3	3.8E-174	5.1E-173	D8	
STY0701	putative hydrolase C-terminus	5.I Unknown	8.1	13.1	2.0E-178	2.8E-177	D8	
STY1193	conserved hypothetical protein	5.H Hypothetical protein	8.1	11.5	4.2E-167	5.3E-166	D8	
STY0678	cold shock-like protein cspE	2. Broad regulatory function	8.1	13.6	1.9E-179	2.6E-178	D8	
STY0231	protease DO precursor; heat shock protein HtrA	3.B.3 Degradation of proteins, peptides, glycoproteins	8.1	13.2	9.5E-178	1.3E-176	D8	
pilO	putative pilus assembly protein	4.I Pathogenesis	8.1	12.4	1.5E-173	2.0E-172	D8	
STY2402	putative lipoprotein	3.C.1 Membranes lipoprotein	8.1	11.0	2.8E-163	3.4E-162	D8	
STY0480	conserved hypothetical protein	5.H Hypothetical protein	8.1	12.0	1.1E-170	1.4E-169	D8	
bigA	pseudogene	3.C.2 Surface polysacchar	8.1	13.8	1.1E-179	1.6E-178	D8	
orgAb	oxygen-regulated invasion protein	4.I Pathogenesis	8.1	13.2	1.6E-177	2.2E-176	D8	
STY2819	putative oxidoreductase	5.I Unknown	8.0	12.9	2.7E-175	3.6E-174	D8	
STY4756	putative exported protein	3.C.1 Membranes lipoprotein	8.0	14.4	1.8E-180	2.7E-179	D8	
STY2849	ClpB protein (heat shock protein f84.1)	3.B.3 Degradation of proteins, peptides, glycoproteins	8.0	13.0	1.0E-175	1.3E-174	D8	
cstA	probable carbon starvation protein	4.A Transport/binding proteins	8.0	13.3	8.9E-177	1.2E-175	D8	

rcsC	sensor protein RcsC	2. Broad regulatory function	8.0	13.9	1.6E-178	2.2E-177	D8	
STY3319	probable hydrogenase-2 cytochrome b subunit	1.B.7.b Anaerobic Respiration	8.0	11.9	3.4E-167	4.3E-166	D8	
STY4484	formate dehydrogenase H	1.B.8 Fermentation	8.0	13.3	2.3E-175	3.1E-174	D8	
mgtB	Magnesium transport ATPase, P-type 2	4.A Transport/binding proteins	8.0	14.0	3.5E-177	4.8E-176	D8	
STY4568	possible exported protein	4.I Pathogenesis	7.9	12.2	1.8E-168	2.3E-167	D8	
STY4256	sn-Glycerol-3-phosphate transport system permease protein	4.A.3 Transport Carbohydes, organic acids and alcohols	7.9	10.6	2.1E-155	2.4E-154	D8	
STY2367	putative membrane protein	3.C.1 Membranes lipoprotein	7.9	12.1	1.1E-166	1.3E-165	D8	D1
STY2843	conserved hypothetical protein	5.H Hypothetical protein	7.9	10.1	2.3E-146	2.4E-145	D8	
STY1793	catalase HP11	4.G Detoxification	7.8	12.1	2.9E-166	3.6E-165	D8	
STY2760	putative exported protein	4.I Pathogenesis	7.8	13.6	1.6E-172	2.2E-171	D8	
ybiR	putative membrane protein	3.C.1 Membranes lipoprotein	7.8	11.2	1.4E-158	1.7E-157	D8	
STY0112	DNA polymerase II	3.A.7 DNA replication, repair, restriction, modification	7.8	12.5	5.5E-167	6.9E-166	D8	
STY3093	hypothetical protein	5.I Unknown	7.7	13.3	2.2E-169	2.8E-168	D8	
STY1228	conserved hypothetical protein	5.H Hypothetical protein	7.7	12.5	5.5E-166	6.8E-165	D8	
STY1921	probable cytochrome oxidase subunit II	1.B.7.c Electron Transport	7.7	10.5	2.0E-151	2.3E-150	D8	
STY2756	conserved hypothetical protein	4.I Pathogenesis	7.7	11.9	1.6E-162	1.9E-161	D8	
pqaB	melittin resistance protein PqaB	5.D Drug/Analogue sensitivity	7.7	11.6	4.5E-160	5.3E-159	D8	
STY1281	putative calcium/proton antiporter	4.A.2 Transport Cations	7.7	12.8	1.8E-166	2.2E-165	D8	
STY4937	inner membrane protein CreD	5.A Laterally acquired elements	7.7	12.3	6.4E-164	7.8E-163	D8	
STY3592	putative hydrolase	5.I Unknown	7.7	10.2	6.1E-148	6.5E-147	D8	
ybhP	conserved hypothetical protein	5.H Hypothetical protein	7.7	9.0	3.2E-112	2.8E-111	D8	
STY1346	osmotically inducible lipoprotein B precursor	5.F Adaptions and atypical conditions	7.7	12.9	9.1E-167	1.1E-165	D8	
STY2611	conserved hypothetical protein	5.H Hypothetical protein	7.7	9.3	2.1E-122	2.0E-121	D8	
STY3405	probable membrane transport protein	4.A Transport/binding proteins	7.7	12.0	2.8E-161	3.2E-160	D8	
STY3044	DeoR-family transcriptional regulator	2. Broad regulatory function	7.7	8.6	6.1E-100	5.2E-99	D8	
slrP	pseudogene	4.I Pathogenesis	7.7	13.5	1.2E-167	1.5E-166	D8	
STY2124	chemotaxis protein CheZ	4.D Chemotaxis and mobility	7.6	10.0	1.6E-144	1.7E-143	D8	
STY0358	hypothetical protein DinP (DNA damage-inducible protein)	5.I Unknown	7.6	12.5	1.9E-163	2.3E-162	D8	
frdA	fumarate reductase, flavoprotein subunit	1.B.7.b Anaerobic Respiration	7.6	12.3	4.2E-162	5.0E-161	D8	
STY3936	putative membrane transport protein	4.A Transport/binding proteins	7.6	11.7	1.8E-158	2.0E-157	D8	
STY2190	colanic acid capsular biosynthesis activation protein A	3.C.2 Surface polysacchar	7.6	11.9	9.4E-160	1.1E-158	D8	
STY3538	possible GntR-family transcriptional regulator	2. Broad regulatory function	7.6	10.8	4.6E-151	5.1E-150	D8	
STY3549	possible exported protein	3.C.1 Membranes lipoprotein	7.6	12.8	1.3E-163	1.5E-162	D8	
STY0517	conserved hypothetical protein	5.H Hypothetical protein	7.6	13.1	8.9E-165	1.1E-163	D8	
STY2158	cell-division regulatory protein	4.C Cell division	7.5	11.1	5.2E-151	5.8E-150	D8	
ampH	penicillin-binding protein AmpH	3.C.4 Murein sacculus and peptidoglycan	7.5	12.8	9.0E-162	1.1E-160	D8	

STY4562	hypothetical protein	4.1 Pathogenesis	7.5	13.6	5.4E-164	6.5E-163	D8	
STY0168	AmpD protein (anhydro-N-acetylmuramyl-tripeptide amidase)	5.1 Unknown	7.5	8.9	2.0E-110	1.8E-109	D8	
STY0395	AraC-family transcriptional regulator	2. Broad regulatory function	7.5	10.4	4.6E-145	4.8E-144	D8	D1
STY1304	periplasmic oligopeptide-binding protein precursor	4.A.6 Transport Other	7.5	13.1	2.6E-161	3.1E-160	D8	
STY4468	lysR family regulatory protein	2. Broad regulatory function	7.4	12.1	4.2E-156	4.8E-155	D8	
STY2804	transcriptional activator cadC	2. Broad regulatory function	7.4	14.8	4.9E-164	6.0E-163	D8	
STY3765	hypothetical protein	5.1 Unknown	7.4	12.3	6.4E-157	7.4E-156	D8	
treC	trehalose-6-phosphate hydrolase	1.A.1 Degradation of carbohydrate	7.4	11.3	9.2E-150	1.0E-148	D8	
STY3826	rhamnulokinase	1.A.1 Degradation of carbohydrate	7.4	11.1	1.4E-147	1.5E-146	D8	
bisC	biotin sulfoxide reductase	#N/A	7.4	12.0	8.8E-154	9.9E-153	D8	
STY3246	endonuclease I	3.B.2 Degradation of DNA	7.4	10.5	2.8E-143	2.9E-142	D8	
prgH	pathogenicity 1 island effector protein	4.1 Pathogenesis	7.3	12.6	1.0E-156	1.2E-155	D8	
pduE	diol dehydratase small subunit	1.A.1 Degradation of carbohydrate	7.3	8.4	1.1E-93	8.9E-93	D8	
STY0259	lysine decarboxylase	1.B.2 Pyruvate dehydrogenase	7.3	12.3	1.5E-154	1.7E-153	D8	
STY4136	xylose operon regulatory protein	2. Broad regulatory function	7.3	11.0	2.1E-146	2.2E-145	D8	
sspH2	secreted effector protein	4.1 Pathogenesis	7.3	11.8	9.2E-152	1.0E-150	D8	
tcfD	putative fimbril protein	4.1 Pathogenesis	7.3	12.4	2.1E-154	2.4E-153	D8	
fimF	fimbria-like protein FimF precursor	3.C.3 Surface structure	7.3	9.7	9.4E-135	9.4E-134	D8	
STY1753	conserved hypothetical protein	5.H Hypothetical protein	7.3	10.9	6.4E-145	6.7E-144	D8	
melB	melibiose carrier protein	4.A.3 Transport Carbohydrates, organic acids and alcohols	7.3	10.9	5.2E-145	5.4E-144	D8	
vexE	Vi polysaccharide export protein	4.1 Pathogenesis	7.2	13.0	1.3E-154	1.5E-153	D8	
shdA	pseudogene	4.1 Pathogenesis	7.2	13.5	1.4E-155	1.6E-154	D8	
STY4001	putative carbohydrate kinase	3.A.10 Polysaccharides (Cytoplasmic)	7.2	12.9	8.5E-154	9.6E-153	D8	
STY0303	probable lipoprotein	4.1 Pathogenesis	7.2	9.3	2.5E-121	2.3E-120	D8	M3
STY1276	putative secreted protein	3.C.1 Membranes lipoprotein	7.2	10.1	7.8E-136	7.8E-135	D8	
yliB	putative ABC transporter periplasmic binding protein	4.A Transport/binding proteins	7.2	11.8	4.7E-148	5.1E-147	D8	
STY2311	pseudogene	3.C.2 Surface polysacchar	7.2	11.9	1.8E-148	2.0E-147	D8	
STY2035	putative bacteriophage protein	#N/A	7.1	9.2	4.8E-118	4.4E-117	D8	
STY3096	sensor protein	2. Broad regulatory function	7.1	14.2	4.3E-155	4.9E-154	D8	
STY1253	putative TetR-family regulatory protein	2. Broad regulatory function	7.1	10.9	1.3E-141	1.3E-140	D8	
STY2494	putative two-component system sensor kinase	2. Broad regulatory function	7.1	12.8	1.2E-151	1.3E-150	D8	
STY3771	pseudogene	1.C.5 Sulphur Metabolism	7.1	12.5	9.0E-150	9.9E-149	D8	D1
lysR	transcriptional activator protein LysR	1.D.2 Aspartate Family	7.1	10.3	2.0E-135	2.0E-134	D8	
scsB	membrane protein, suppressor for copper-sensitivity B precursor	4.G Detoxification	7.1	11.2	2.6E-142	2.7E-141	D8	

tviE	Vi polysaccharide biosynthesis protein TviE, Glycosyl transferases group 1	4.I Pathogenesis	7.0	13.1	9.1E-150	1.0E-148	D8	
vexA	Vi polysaccharide export protein	4.I Pathogenesis	7.0	13.5	7.2E-151	7.9E-150	D8	
STY3671	possible lipoprotein	5.I Unknown	7.0	12.9	4.4E-149	4.8E-148	D8	
waaB	lipopolysaccharide 1,6-galactosyltransferase	3.C.2 Surface polysacchar	7.0	13.4	5.0E-150	5.6E-149	D8	
STY2485	probable nitrate reductase	1.B.7.b Anaerobic Respiration	7.0	11.7	2.0E-143	2.1E-142	D8	
STY2875	large repetitive protein	4.I Pathogenesis	7.0	13.6	1.1E-149	1.2E-148	D8	
STY4075	hypothetical protein	5.I Unknown	7.0	11.5	1.1E-141	1.1E-140	D8	
phoN	nonspecific acid phosphatase precursor	1.C Central Intermediary metabolism	7.0	11.5	1.8E-141	1.9E-140	D8	
STY0710	ABC transporter periplasmic binding protein (glutamate/aspartate?)	4.A Transport/binding proteins	6.9	9.9	2.0E-128	1.9E-127	D8	
STY4236	putative membrane protein	3.C.1 Membranes lipoprotein	6.9	10.0	3.7E-129	3.6E-128	D8	
STY1186	glucans biosynthesis protein	5.F Adaptions and atypical conditions	6.9	12.6	1.9E-145	2.0E-144	D8	
STY0159	putative lysR-family transcriptional regulator	2. Broad regulatory function	6.9	10.4	4.3E-132	4.3E-131	D8	
STY0439	maltodextrin glucosidase	1.A.1 Degradation of carbohydrate	6.9	10.3	8.0E-132	7.9E-131	D8	
STY4268	gluconate utilization operon repressor	1.B.6 Entner-Doudoroff pathway	6.9	10.3	1.8E-131	1.8E-130	D8	
STY2775	pseudogene	1.B.7.b Anaerobic Respiration	6.9	11.5	1.1E-139	1.1E-138	D8	
tldD	TldD protein	5.I Unknown	6.9	11.1	1.2E-136	1.2E-135	D8	
STY0180	putative exported protein	3.C.1 Membranes lipoprotein	6.9	10.8	2.1E-134	2.0E-133	D8	
STY3860	putative membrane permease	4.A Transport/binding proteins	6.9	10.9	5.5E-135	5.5E-134	D8	
STY1496	conserved hypothetical protein	5.H Hypothetical protein	6.8	14.0	1.6E-146	1.7E-145	D8	
STY4824	hypothetical protein	5.H Hypothetical protein	6.8	14.0	2.5E-146	2.7E-145	D8	
STY3721	thiamine biosynthesis protein	1.G.8 Thiamine	6.8	12.8	7.3E-143	7.6E-142	D8	
yifB	pseudogene	5.H Hypothetical protein	6.8	16.7	9.1E-147	9.7E-146	D8	
STY3923	conserved hypothetical protein	5.H Hypothetical protein	6.8	13.9	2.0E-144	2.1E-143	D8	
STY3796	putative ABC transporter ATP-binding protein	4.A Transport/binding proteins	6.8	11.5	8.0E-137	8.1E-136	D8	
STY4060	RNase PH	3.B.1 Degradation of RNA	6.8	12.2	8.9E-140	9.1E-139	D8	
STY4785	hypothetical protein	5.I Unknown	6.8	10.3	5.6E-128	5.4E-127	D8	
STY4804	carbamate kinase	1.A.2 Degradation of amino acids	6.7	10.2	3.3E-127	3.2E-126	D8	
STY0616	hypothetical protein	5.H Hypothetical protein	6.7	9.4	8.9E-120	8.3E-119	D8	
STY3594	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase	1.D.2 Aspartate Family	6.7	14.8	3.3E-145	3.5E-144	D8	
STY2835	conserved hypothetical protein	5.H Hypothetical protein	6.7	12.1	4.5E-138	4.6E-137	D8	
STY2859	putative membrane protein	3.C.1 Membranes lipoprotein	6.7	12.3	1.6E-138	1.6E-137	D8	
yigL	conserved hypothetical protein	5.H Hypothetical protein	6.6	10.5	3.5E-127	3.4E-126	D8	
STY1016	exonuclease	5.A Laterally acquired elements	6.6	8.4	7.9E-95	6.7E-94	D8	D1
steC	periplasmic fimbrial chaperone	3.C.3 Surface structure	6.6	10.0	1.1E-122	1.0E-121	D8	

STY0384	pseudogene	3.C Cell envelope	6.6	9.6	2.1E-118	1.9E-117	D8	
STY1669	endonuclease III	3.B.2 Degradation of DNA	6.6	8.3	9.1E-89	7.6E-88	D8	
STY3400	conserved hypothetical protein	5.H Hypothetical protein	6.6	10.7	5.4E-128	5.2E-127	D8	
STY0973	formate acetyltransferase I	1.B.7.b Anaerobic Respiration	6.6	11.3	2.9E-131	2.9E-130	D8	
STY2213	hypothetical protein	5.I Unknown	6.6	9.1	3.7E-113	3.3E-112	D8	
STY0081	probable crotonobetaine/carnitine-CoA ligase	1.A.1 Degradation of carbohydrate	6.6	10.8	7.2E-128	6.9E-127	D8	
STY3443	PTS system, galactitol-specific IIC component	4.A.3 Transport Carbohydrates, organic acids and alcohols	6.5	10.0	4.0E-121	3.7E-120	D8	
STY0167	nicotinate-nucleotide pyrophosphorylase	1.G.7 Pyridine nucleotide	6.5	8.9	3.7E-111	3.3E-110	D8	
STY0568	probable metabolite transport protein	4.A Transport/binding proteins	6.5	8.4	1.1E-93	9.6E-93	D8	
STY4459	large repetitive protein	4.I Pathogenesis	6.5	13.2	5.5E-135	5.5E-134	D8	
STY2526	Ais protein	5.I Unknown	6.5	12.0	1.8E-131	1.8E-130	D8	
STY3794	putative ABC transporter, membrane component	4.A Transport/binding proteins	6.4	10.9	3.6E-125	3.4E-124	D8	
STY2397	D-lactate dehydrogenase	1.B.7.a Aerobic Respiration	6.4	10.0	2.2E-118	2.0E-117	D8	
STY3403	possible oxidoreductase	5.I Unknown	6.4	10.1	1.9E-118	1.7E-117	D8	
STY2387	conserved hypothetical protein	5.H Hypothetical protein	6.4	9.9	6.3E-116	5.7E-115	D8	
STY0855	putative ATP-dependent RNA helicase rhIE	5.I Unknown	6.4	10.4	5.4E-121	5.1E-120	D8	
STY2511	glycerophosphoryl diester phosphodiesterase periplasmic precursor	1.C Central Intermediary metabolism	6.3	12.2	5.6E-129	5.4E-128	D8	
STY3406	putative membrane protein	3.C.1 Membranes lipoprotein	6.3	11.1	6.0E-124	5.6E-123	D8	
STY0943	ATP-dependent Clp protease ATP-binding subunit ClpA	3.B.3 Degradation of proteins, peptides, glycoproteins	6.3	11.3	1.7E-125	1.6E-124	D8	
STY3670	possible lipoprotein	5.I Unknown	6.3	11.6	2.9E-126	2.8E-125	D8	
STY3167	probable amino acid transport protein	4.A.1 Transport amino acid and amines	6.3	12.6	4.7E-129	4.6E-128	D8	
cobD	putative aminotransferase CobD	1.G.13 Cobalamin	6.3	9.9	2.7E-115	2.4E-114	D8	
STY2625	putative membrane protein	3.C.1 Membranes lipoprotein	6.3	10.3	3.7E-117	3.3E-116	D8	
STY4197	hypothetical luxR-family transcriptional regulator	2. Broad regulatory function	6.2	14.1	6.8E-131	6.7E-130	D8	
STY1312	cardiolipin synthetase	3.A.11 Phospholipids	6.2	10.3	3.4E-116	3.1E-115	D8	D1
STY3380	adenyl-transferase	3.A.8 Protein translation and modification	6.2	11.1	1.9E-120	1.8E-119	D8	
STY4664	putative DNA helicase	4.I Pathogenesis	6.2	11.7	1.3E-122	1.3E-121	D8	
STY3404	possible drug efflux protein	5.D Drug/Analogue sensitivity	6.2	10.9	6.9E-119	6.4E-118	D8	
STY0426	hypothetical ROK-family protein	5.I Unknown	6.2	8.8	2.8E-100	2.4E-99	D8	
STY3295	possible amino acid transport protein	4.A Transport/binding proteins	6.2	10.0	1.8E-112	1.6E-111	D8	
STY0582	phosphoribosylaminoimidazole carboxylase catalytic subunit	1.F.1 Purine ribonucleotide biosynthesis	6.2	8.4	5.6E-94	4.7E-93	D8	
STY4173	putative amino acid permease	4.A.1 Transport amino acid and amines	6.2	12.5	1.4E-124	1.3E-123	D8	
STY4782	putative membrane protein	3.C.1 Membranes lipoprotein	6.1	8.8	1.3E-100	1.2E-99	D8	D1
STY1448	putative phosphotransferase enzyme	4.A Transport/binding proteins	6.1	7.6	9.6E-65	7.6E-64	D8	
citF2	citrate lyase alpha chain	1.C Central Intermediary metabolism	6.1	9.2	9.3E-104	8.1E-103	D8	



STY3435	tagatose-bisphosphate aldolase	1.A.1 Degradation of carbohydrate	6.1	10.6	7.4E-115	6.6E-114	D8	
tvIC	Vi polysaccharide biosynthesis protein, epimerase	4.I Pathogenesis	6.1	11.6	1.5E-119	1.4E-118	D8	
STY1428	ATP-dependent helicase HrpA	3.A.7 DNA replication, repair, restriction, modification	6.1	11.2	1.4E-117	1.3E-116	D8	
STY4212	universal stress protein A	5.F Adaptions and atypical conditions	6.1	9.7	9.6E-108	8.4E-107	D8	
STY1763	3-deoxy-D-arabinoheptulosonate 7-phosphate synthase	1.D.4 Aromatic Amino Acid	6.0	8.3	1.7E-91	1.4E-90	D8	
STY0111	probable ATP-dependent helicase HepA	3.A.9 RNA synthesis, modification	6.0	10.8	5.4E-114	4.8E-113	D8	
STY1425	putative secreted protein	3.C.1 Membranes lipoprotein	6.0	9.7	1.6E-105	1.4E-104	D8	
STY3837	putative lipoprotein	3.C.1 Membranes lipoprotein	6.0	11.3	4.2E-116	3.8E-115	D8	
ratB	pseudogene	4.I Pathogenesis	5.9	11.0	3.8E-113	3.4E-112	D8	
hflC	HflC protein	5.I Unknown	5.9	8.6	3.7E-92	3.1E-91	D8	
STY0043	possible sulfatase regulatory protein	1.C.5 Sulphur Metabolism	5.9	13.5	5.2E-118	4.8E-117	D8	
pduG	PduG protein	1.A.1 Degradation of carbohydrate	5.8	8.8	2.4E-92	2.0E-91	D8	
dcuS	two-component sensor kinase	2. Broad regulatory function	5.8	10.1	3.5E-105	3.0E-104	D8	
tcfA	putative fimbrial protein	4.I Pathogenesis	5.8	9.5	3.0E-99	2.5E-98	D8	
STY3066	conserved hypothetical protein	5.H Hypothetical protein	5.8	8.3	1.2E-85	1.0E-84	D8	
pmrB	two-component sensor kinase	2. Broad regulatory function	5.7	9.8	8.6E-102	7.5E-101	D8	
allB	putative allantoinase	1.B.9 ATP-proton motive factor	5.7	10.4	2.0E-105	1.8E-104	D8	
STY4475	cytochrome c552 precursor	1.B.7.c Electron Transport	5.7	9.7	2.0E-99	1.7E-98	D8	
tvIB	Vi polysaccharide biosynthesis protein, UDP-glucose/GDP-mannose dehydrogenase	4.I Pathogenesis	5.7	13.2	3.5E-112	3.1E-111	D8	
STY1742	pseudogene	4.I Pathogenesis	5.6	9.7	3.7E-97	3.1E-96	D8	
STY3942	recF protein	3.A.7 DNA replication, repair, restriction, modification	5.6	10.1	2.5E-100	2.2E-99	D8	
STY2977	hydrogenase isoenzymes formation protein HypB	1.B.8 Fermentation	5.6	9.4	2.3E-94	1.9E-93	D8	D1
samA	pseudogene	#N/A	5.6	14.6	2.7E-111	2.4E-110	D8	
STY1423	putative exported protein	3.C.1 Membranes lipoprotein	5.6	10.6	1.3E-102	1.1E-101	D8	
STY0131	3-isopropylmalate dehydrogenase	1.D.6 Pyruvate Family	5.5	9.7	8.4E-96	7.2E-95	D8	
STY3223	putative membrane protein	3.C.1 Membranes lipoprotein	5.5	10.4	6.4E-100	5.5E-99	D8	
tsaC	outer membrane fimbrial usher protein	4.I Pathogenesis	5.5	10.2	9.3E-99	8.0E-98	D8	
STY2525	conserved hypothetical protein	5.H Hypothetical protein	5.4	9.7	2.7E-94	2.3E-93	D8	
STY2617	conserved hypothetical protein	5.I Unknown	5.4	8.9	1.1E-86	8.8E-86	D8	
STY4043	sodium:galactoside family symporter	4.A.3 Transport Carbohydes, organic acids and alcohols	5.3	8.8	5.0E-83	4.2E-82	D8	
tvID	Vi polysaccharide biosynthesis protein	4.I Pathogenesis	5.3	13.1	2.7E-102	2.3E-101	D8	
invA	possible secretory protein (associated with virulence)	4.I Pathogenesis	5.3	8.3	1.5E-76	1.2E-75	D8	D1
STY1315	putative acyl-coA hydrolase	5.I Unknown	5.3	8.7	1.7E-80	1.4E-79	D8	
STY2801	putative 3-phenylpropionate permease	4.A.3 Transport Carbohydes, organic acids and alcohols	5.3	8.5	1.7E-77	1.4E-76	D8	
wzc	putative tyrosine-protein kinase	3.A.8 Protein translation and modification	5.2	8.9	2.9E-81	2.4E-80	D8	
dsbA	disulfide isomerase	3.A.8 Protein translation and modification	5.1	9.0	6.2E-81	5.1E-80	D8	

glgB	1,4-alpha-glucan branching enzyme	3.A.10 Polysaccharides (Cytoplasmic)	5.1	10.6	4.8E-91	4.0E-90	D8	
vexB	Vi polysaccharide export inner-membrane protein (ABC-2 type transporter)	4.I Pathogenesis	5.1	10.2	8.1E-89	6.8E-88	D8	
STY2216	putative inner membrane protein	3.C.1 Membranes lipoprotein	5.1	8.6	8.5E-75	6.8E-74	D8	
STY4856	conserved hypothetical protein	5.H Hypothetical protein	5.0	9.3	4.0E-81	3.3E-80	D8	
STY3326	putative membrane protein	3.C.1 Membranes lipoprotein	5.0	9.1	7.0E-78	5.7E-77	D8	
mgtC	conserved hypothetical protein	5.H Hypothetical protein	5.0	12.6	3.7E-92	3.1E-91	D8	
STY4222	putative membrane protein	3.C.1 Membranes lipoprotein	4.9	9.9	1.0E-82	8.5E-82	D8	
mrr	mrr restriction system protein	3.A.7 DNA replication, repair, restriction, modification	4.9	8.2	2.3E-66	1.8E-65	D8	
hsdR	subunit R of type I restriction-modification system	3.A.7 DNA replication, repair, restriction, modification	4.9	9.4	1.3E-78	1.0E-77	D8	
STY2876	putative type I secretion protein	4.I Pathogenesis	4.9	8.3	4.7E-67	3.7E-66	D8	
mgtA	Mg(2+) transport ATPase, P-type	4.A.2 Transport Cations	4.9	9.6	8.5E-80	6.9E-79	D8	
STY2171	cytoplasmic alpha-amylase	3.B.4 Degradations of polysaccharides	4.8	13.1	1.1E-90	9.4E-90	D8	
staA	putative fimbrial protein	3.C.3 Surface structure	4.8	6.9	2.5E-42	1.9E-41	D8	
STY0085	FixA protein	1.A.1 Degradation of carbohydrate	4.8	8.2	1.6E-64	1.3E-63	D8	D1
ytfM	putative exported protein	3.C.1 Membranes lipoprotein	4.7	9.5	5.3E-75	4.2E-74	D8	
stdB	probable outer membrane fimbrial usher protein	3.C.3 Surface structure	4.7	9.6	3.5E-75	2.8E-74	D8	
STY1460	putative peptidase	3.B.3 Degradation of proteins, peptides, glycoproteins	4.6	8.9	6.7E-69	5.4E-68	D8	
STY3451	conserved hypothetical protein	5.H Hypothetical protein	4.6	6.4	4.0E-31	3.0E-30	D8	
STY0057	probable FkbB-type 16 kD peptidyl-prolyl cis-trans isomerase	3.A.8 Protein translation and modification	4.6	5.2	1.1E-18	7.7E-18	D8	
STY1202	conserved hypothetical protein	5.H Hypothetical protein	4.6	7.3	5.3E-46	4.1E-45	D8	
STY2825	DNA repair protein RecO	3.A.7 DNA replication, repair, restriction, modification	4.6	7.3	2.4E-45	1.9E-44	D8	D1
STY4467	putative sodium/hydrogen exchanger family protein	4.A Transport/binding proteins	4.5	8.3	4.8E-60	3.8E-59	D8	
STY3083	conserved hypothetical protein	5.H Hypothetical protein	4.5	12.0	3.6E-79	2.9E-78	D8	
STY0197	conserved hypothetical protein	5.H Hypothetical protein	4.5	8.4	2.5E-60	2.0E-59	D8	
STY3684	putative regulatory protein	5.I Unknown	4.5	7.3	1.5E-44	1.2E-43	D8	D1
STY0768	pseudogene	5.H Hypothetical protein	4.4	3.2	4.7E-09	2.9E-08	D8	
slsA	conserved hypothetical protein	5.H Hypothetical protein	4.4	8.5	1.8E-61	1.4E-60	D8	
yjfl	putative membrane protein	3.C.1 Membranes lipoprotein	4.4	7.4	1.3E-43	9.9E-43	D8	D1
STY4424	maltose transport inner membrane protein	4.A.3 Transport Carbohydrates, organic acids and alcohols	4.3	9.2	8.5E-64	6.7E-63	D8	
STY2981	transcriptional activator of the formate hydrogenlyase system	2. Broad regulatory function	4.2	8.3	3.8E-54	3.0E-53	D8	
STY3307	D-mannonate oxidoreductase	1.A.1 Degradation of carbohydrate	4.1	7.4	1.5E-40	1.2E-39	D8	D1
STY0050	putative transport protein	4.A Transport/binding proteins	4.1	11.4	7.9E-68	6.3E-67	D8	
STY0319	Rhs-family protein	4.I Pathogenesis	4.1	7.4	8.1E-40	6.1E-39	D8	
STY3129	N-acetylmuramoyl-L-alanine amidase	3.C.4 Murein sacculus and peptidoglycan	4.1	8.4	1.8E-52	1.4E-51	D8	

STY1933	conserved hypothetical protein	5.H Hypothetical protein	4.1	8.1	1.3E-48	1.0E-47	D8	
STY0473	ApbA	1.F.2 Pyrimidine ribonucle	4.0	6.6	2.4E-28	1.8E-27	D8	
STY2083	conserved hypothetical protein	5.H Hypothetical protein	4.0	9.6	4.0E-60	3.1E-59	D8	
STY1409	putative membrane transport protein	4.A Transport/binding proteins	4.0	6.2	2.1E-23	1.5E-22	D8	
STY0896	D-alanyl-D-alanine carboxypeptidase (penicillin-binding protein 6 precursor)	3.C.4 Murein sacculus and peptidoglycan	3.9	8.7	3.9E-51	3.0E-50	D8	
STY1804	conserved hypothetical protein	5.H Hypothetical protein	3.9	7.6	1.5E-39	1.1E-38	D8	
STY4089	putative exported protein	3.C.1 Membranes lipoprotein	3.9	8.0	7.6E-45	5.9E-44	D8	
STY3494	conserved hypothetical protein	5.H Hypothetical protein	3.9	6.6	8.5E-26	6.2E-25	D8	
STY1593	hypothetical protein	5.I Unknown	3.8	6.2	4.7E-22	3.4E-21	D8	
STY0923	arginine-binding periplasmic protein 1 precursor	4.A.1 Transport amino acid and amines	3.8	7.1	8.2E-33	6.2E-32	D8	
STY0484	cytochrome o ubiquinol oxidase subunit I	1.B.7.a Aerobic Respiration	3.8	5.9	3.1E-19	2.2E-18	D8	D1
STY3603	conserved hypothetical protein	5.H Hypothetical protein	3.8	5.9	9.7E-20	6.8E-19	D8	
STY3355	probable two-component system sensor histidine kinase	2. Broad regulatory function	3.8	6.7	2.1E-26	1.6E-25	D8	
citG	CitG protein	5.I Unknown	3.7	5.6	1.6E-16	1.1E-15	D8	
yrfE	putative NUDIX hydrolase	5.I Unknown	3.7	5.8	1.3E-17	8.7E-17	D8	
STY3123	possible lipoprotein	3.C.1 Membranes lipoprotein	3.6	6.0	1.1E-18	7.5E-18	D8	
STY2879	putative reverse transcriptase	4.I Pathogenesis	3.5	7.5	1.5E-32	1.2E-31	D8	
STY2149	putative exported protein	3.C.1 Membranes lipoprotein	3.5	7.2	4.6E-29	3.5E-28	D8	
STY0004	threonine synthase	1.D.2 Aspartate Family	3.5	6.9	5.2E-26	3.8E-25	D8	
STY3248	glutathione synthetase	1.G.10 Thioredoxin	3.5	3.8	1.5E-07	8.9E-07	D8	
misL	pseudogene	4.I Pathogenesis	3.5	9.2	4.7E-46	3.7E-45	D8	
STY2513	anaerobic glycerol-3-phosphate dehydrogenase subunit A	1.B.7.b Anaerobic Respiration	3.5	7.0	2.5E-26	1.8E-25	D8	
STY4470	putative membrane protein	3.C.1 Membranes lipoprotein	3.4	4.1	6.0E-08	3.6E-07	D8	
STY0481	cytochrome o ubiquinol oxidase C subunit	1.B.7.a Aerobic Respiration	3.4	4.6	9.6E-10	6.0E-09	D8	M3
ugd	UDP-glucose 6-dehydrogenase	1.C.3 Sugar Nuclotides	3.3	9.0	8.8E-42	6.7E-41	D8	
STY2763a	conserved hypothetical protein	5.H Hypothetical protein	3.3	6.5	3.7E-20	2.6E-19	D8	
STY3165	possible regulatory protein	2. Broad regulatory function	3.3	6.6	5.8E-21	4.1E-20	D8	
STY2716	putative exported protein	3.C.1 Membranes lipoprotein	3.3	9.7	5.5E-44	4.3E-43	D8	
STY2771	nucleoside diphosphate kinase (ndk)	1.F.1 Purine ribonucleotide biosynthesis	3.2	4.7	3.8E-09	2.4E-08	D8	
STY2951	conserved hypothetical protein	5.H Hypothetical protein	3.0	5.4	7.0E-11	4.6E-10	D8	
treR	trehalose operon repressor	2. Broad regulatory function	2.9	9.0	3.3E-34	2.5E-33	D8	
STY3397	pseudogene	5.I Unknown	2.9	6.8	1.3E-18	9.2E-18	D8	
iroN	TonB-dependent outer membrane siderophore receptor protein	4.I Pathogenesis	2.9	8.1	1.7E-28	1.3E-27	D8	
STY0639	isochorismate synthase EntC	1.G.14 Iron uptake and storage	2.9	4.1	2.3E-06	1.2E-05	D8	
STY1503	putative hydrolase	5.I Unknown	2.9	6.4	8.9E-15	6.0E-14	D8	

STY1392	hypothetical protein	5.I Unknown	2.8	8.6	2.8E-30	2.1E-29	D8	
STY1666	putative membrane protein	3.C.1 Membranes lipoprotein	2.8	4.8	1.5E-07	8.7E-07	D8	
STY1928	conserved hypothetical protein	5.H Hypothetical protein	2.7	6.8	1.3E-16	8.9E-16	D8	
sthE	pseudogene	3.C.3 Surface structure	2.7	6.5	2.7E-14	1.8E-13	D8	
STY1317	putative membrane protein	3.C.1 Membranes lipoprotein	2.7	6.9	7.2E-17	4.9E-16	D8	
STY3268	nucleoside permease	4.A.6 Transport Other	2.7	6.9	1.5E-16	1.0E-15	D8	
prkB	phosphoribulokinase	1.C Central Intermediary metabolism	2.7	6.3	1.6E-12	1.1E-11	D8	
STY1458	putative benzoate membrane transport protein	5.I Unknown	2.6	5.3	2.2E-08	1.4E-07	D8	
STY2908	putative membrane protein	3.C.1 Membranes lipoprotein	2.6	4.6	3.4E-06	1.8E-05	D8	
yodD	conserved hypothetical protein	5.H Hypothetical protein	2.6	4.8	1.2E-06	6.8E-06	D8	
STY0298	pseudogene	4.I Pathogenesis	2.6	5.6	4.6E-09	2.9E-08	D8	
STY1089	conserved hypothetical protein	5.H Hypothetical protein	2.6	6.7	2.4E-14	1.6E-13	D8	
STY1683	putative oxidoreductase	5.I Unknown	2.6	4.5	8.3E-06	4.2E-05	D8	
STY2268	pseudogene	3.C.4 Murein sacculus and peptidoglycan	2.5	5.4	2.5E-08	1.5E-07	D8	
STY0935	conserved hypothetical protein	5.H Hypothetical protein	2.5	5.9	3.2E-10	2.1E-09	D8	
STY3861	putative membrane permease	4.A Transport/binding proteins	2.5	7.3	5.9E-17	4.1E-16	D8	
STY4000	putative PTS system IIC component	4.A.3 Transport Carbohydes, organic acids and alcohols	2.5	9.9	4.1E-27	3.0E-26	D8	
waaZ	lipopolysaccharide core biosynthesis protein RfaZ	3.C.2 Surface polysacchar	2.4	9.5	7.5E-26	5.5E-25	D8	
STY2589	putative amino acid transporter	4.A Transport/binding proteins	2.4	6.6	2.1E-12	1.4E-11	D8	
rpoS	RNA polymerase sigma subunit RpoS (sigma-38)	2. Broad regulatory function	2.4	14.7	7.7E-28	5.7E-27	D8	
frdB	fumarate reductase, iron-sulfur protein	1.B.7.b Anaerobic Respiration	2.3	5.5	1.7E-07	1.0E-06	D8	
STY1231	fatty acid/phospholipid synthesis protein PlsX	3.A.11 Phospholipids	2.3	6.7	7.0E-12	4.6E-11	D8	
STY0903	putative membrane protein	3.C.1 Membranes lipoprotein	2.3	6.9	1.4E-12	9.5E-12	D8	
STY4148	putative acetyltransferase	5.I Unknown	2.2	9.7	5.7E-22	4.0E-21	D8	
STY4772	hypothetical protein	5.H Hypothetical protein	2.2	6.2	5.9E-09	3.7E-08	D8	
STY2592	amidophosphoribosyltransferase	1.F.1 Purine ribonucleotide biosynthesis	2.1	6.5	1.3E-09	8.5E-09	D8	
STY4009	putative glycosyl hydrolase	5.I Unknown	2.1	6.1	2.3E-08	1.4E-07	D8	
citT	citrate carrier	4.A Transport/binding proteins	2.1	5.8	3.2E-07	1.9E-06	D8	
kdgR	transcriptional regulator KdgR	2. Broad regulatory function	2.1	5.9	3.6E-07	2.1E-06	D8	
STY0982	integration host factor beta-subunit (IHF-beta)	3.A.7 DNA replication, repair, restriction, modification	2.1	5.4	9.5E-06	4.8E-05	D8	D1M3
wcaD	pseudogene	3.C.2 Surface polysacchar	2.1	6.1	1.1E-07	6.8E-07	D8	
STY3155	galactose operon repressor	1.A.1 Degradation of carbohydrate	2.0	6.0	3.3E-07	1.9E-06	D8	D1
yjeQ	putative membrane protein	3.C.1 Membranes lipoprotein	8.0	3.4	3.4E-08	1.9E-07	M1	
STY3499	RNA polymerase sigma-54 factor (sigma-N)	2. Broad regulatory function	7.3	2.8	4.5E-06	1.8E-05	M1	D1M3
STY2586	putative decarboxylase	5.I Unknown	4.4	4.1	2.5E-10	1.8E-09	M1	M3
STY0103	bis(5'-nucleosyl)-tetrakisphosphate	1.F.4 Salvage of nucleosides and nucleotides	4.3	6.0	6.1E-27	2.0E-25	M1	D1M3

yrfF	putative membrane protein	3.C.1 Membranes lipoprotein	4.3	4.9	3.6E-16	5.2E-15	M1	
STY1006	pseudogene	1.A Degradation	4.3	6.3	9.5E-30	4.0E-28	M1	D1
STY1357	peptide transport system permease protein SapC	4.A.6 Transport Other	4.2	4.0	3.7E-09	2.3E-08	M1	D1M3
pagC	outer membrane invasion protein	4.I Pathogenesis	4.1	4.7	9.2E-14	1.0E-12	M1	
STY3904	glucose inhibited division protein	3.A.7 DNA replication, repair, restriction, modification	4.1	5.5	3.4E-20	6.7E-19	M1	D1M3
STY2567	acetate kinase	1.A.1 Degradation of carbohydrate	4.0	4.3	2.6E-11	2.2E-10	M1	
tufB	elongation factor Tu	3.A.8 Protein translation and modification	4.0	6.6	1.5E-31	6.8E-30	M1	D1D8M3
STY2002	putative membrane protein	3.C.1 Membranes lipoprotein	4.0	4.6	9.1E-13	9.2E-12	M1	
yscR	putative type III secretion protein	4.I Pathogenesis	3.9	4.2	2.2E-10	1.6E-09	M1	
STY1519	membrane transport protein	4.A Transport/binding proteins	3.9	4.9	1.5E-14	1.9E-13	M1	
STY4285	conserved hypothetical protein	5.H Hypothetical protein	3.9	3.7	3.7E-07	1.8E-06	M1	M3
STY1342	conserved hypothetical protein	5.H Hypothetical protein	3.9	6.6	6.9E-30	2.9E-28	M1	
trpS	tryptophanyl-tRNA synthetase	3.A.5 Aminoacyl tRNA synthesis, tRNA modification	3.8	7.1	1.4E-34	8.0E-33	M1	D1D8M3
STY1038	putative bacteriophage protein	5.A Laterally acquired elements	3.8	6.3	5.6E-26	1.7E-24	M1	M3
STY3003	doubtful CDS found within S. typhi pathogenicity island	4.I Pathogenesis	3.7	4.0	7.0E-09	4.3E-08	M1	
STY0788	putative membrane protein	3.C.1 Membranes lipoprotein	3.6	5.2	7.7E-16	1.1E-14	M1	
STY3565	Fis DNA-binding protein	3.A.7 DNA replication, repair, restriction, modification	3.5	6.2	1.6E-23	4.0E-22	M1	
STY1230	50S ribosomal protein L32	3.A.2 Ribosomal protein synthesis, modification	3.3	4.1	2.8E-08	1.6E-07	M1	M3
STY1257	ABC transporter integral membrane subunit	4.A Transport/binding proteins	3.3	5.8	1.2E-18	2.1E-17	M1	
sprB	possible AraC-family transcriptional regulator	4.I Pathogenesis	3.2	5.2	1.3E-13	1.5E-12	M1	
STY1251	NADH dehydrogenase	1.B.7.a Aerobic Respiration	3.2	6.5	7.2E-23	1.7E-21	M1	D1M3
hemK	HemK protein, putative protoporphyrinogen oxidase	5.I Unknown	3.1	4.6	1.5E-10	1.1E-09	M1	M3
yjeT	putative membrane protein	3.C.1 Membranes lipoprotein	3.1	5.3	1.7E-14	2.1E-13	M1	
STY2751	GMP synthase (glutamine-hydrolyzing)	1.F.1 Purine ribonucleotide biosynthesis	2.9	5.8	1.9E-16	2.9E-15	M1	D1M3
STY3703	putative positive regulator of late gene transcription	5.I Unknown	2.9	4.9	4.9E-11	4.0E-10	M1	
STY4585	hypothetical protein	4.I Pathogenesis	2.9	6.2	3.3E-18	5.5E-17	M1	M3
STY0284	ribonuclease H	3.B.1 Degradation of RNA	2.8	5.1	1.2E-11	1.0E-10	M1	
ptsJ	putative transcriptional regulator	2. Broad regulatory function	2.8	4.8	3.5E-10	2.5E-09	M1	D1M3
STY3639	thioredoxin	1.G.10 Thioredoxin	2.8	5.2	1.2E-11	1.1E-10	M1	
STY1178	assembly/transport component in curli production	3.C.3 Surface structure	2.7	5.4	5.1E-13	5.3E-12	M1	
ptsN	nitrogen regulatory IIA protein	2. Broad regulatory function	2.7	6.5	5.0E-19	9.0E-18	M1	M3
STY2619	phosphohistidine phosphatase	2. Broad regulatory function	2.7	6.6	3.4E-19	6.3E-18	M1	M3
STY4163	hypothetical protein	5.I Unknown	2.6	6.8	2.3E-19	4.2E-18	M1	
STY2787	conserved hypothetical protein	5.H Hypothetical protein	2.6	6.0	3.3E-15	4.4E-14	M1	
STY1709	putative pathogenicity island protein	4.I Pathogenesis	2.6	4.4	2.2E-07	1.1E-06	M1	
efp	elongation factor P	3.A.8 Protein translation and modification	2.5	5.5	8.1E-12	7.3E-11	M1	M3

STY0762	pseudogene	3.C.2 Surface polysaccharide and antigen	2.5	4.1	2.4E-06	9.8E-06	M1	
STY0605	putative membrane protein	3.C.1 Membranes lipoprotein	2.5	11.5	8.0E-30	3.3E-28	M1	D8
STY0516	haemolysin expression modulating protein	2. Broad regulatory function	2.4	5.3	6.7E-11	5.4E-10	M1	D1M3
STY3564	conserved hypothetical protein	5.H Hypothetical protein	2.4	9.6	1.3E-26	4.2E-25	M1	
STY2368	hypothetical protein	5.I Unknown	2.4	5.9	4.5E-13	4.7E-12	M1	
argR	arginine repressor	#N/A	2.4	6.1	3.2E-14	3.9E-13	M1	M3
STY1368	peptide transport system permease protein SapB	4.A.6 Transport Other	2.4	4.5	3.9E-07	1.9E-06	M1	M3
gtrA	bactoprenol-linked glucose translocase	3.C.2 Surface polysacchar	2.4	9.4	1.5E-25	4.4E-24	M1	D8
STY3906	ATP synthase protein I.	1.A Degradation	2.4	5.1	2.7E-09	1.7E-08	M1	
STY0970	hypothetical protein	5.I Unknown	2.4	5.5	4.6E-11	3.8E-10	M1	D1M3
yaeP	conserved hypothetical protein	5.H Hypothetical protein	2.3	6.5	2.4E-15	3.3E-14	M1	
STY0529	conserved hypothetical protein	5.H Hypothetical protein	2.3	5.3	5.8E-10	4.0E-09	M1	
STY1369	peptide transport periplasmic protein SapA precursor	4.A.6 Transport Other	2.3	7.2	2.1E-18	3.7E-17	M1	M3
STY3463	polynucleotide phosphorylase	3.A.9 RNA synthesis, modification	2.3	10.3	4.7E-26	1.4E-24	M1	D1D8M3
STY4041	hypothetical protein	4.I Pathogenesis	2.3	6.7	4.0E-16	5.8E-15	M1	
STY0368	probable secreted protein	3.C.1 Membranes lipoprotein	2.3	6.3	1.8E-14	2.3E-13	M1	
STY2944	gamma-glutamylcysteine synthetase	1.G.10 Thioredoxin	2.3	7.8	2.2E-20	4.4E-19	M1	D8
ssaV	putative type III secretion protein	4.I Pathogenesis	2.3	5.9	2.5E-12	2.3E-11	M1	
STY4246	hypothetical protein	5.I Unknown	2.3	4.8	1.1E-07	5.6E-07	M1	M3
tufA	elongation factor Tu	#N/A	2.3	5.3	7.0E-10	4.9E-09	M1	D1
STY0482	cytochrome o ubiquinol oxidase C subunit	1.B.7.a Aerobic Respiration	2.3	6.6	3.8E-15	5.1E-14	M1	
STY3553	rod shape-determining protein	3.C.4 Murein sacculus and peptidoglycan	2.3	4.9	5.1E-08	2.8E-07	M1	
STY3283	bacteriocin immunity protein	4.I Pathogenesis	2.3	4.4	2.1E-06	8.8E-06	M1	
STY3230	putative membrane protein	3.C.1 Membranes lipoprotein	2.3	7.1	6.7E-17	1.0E-15	M1	
gtrB	bactoprenol glucosyl transferase	3.C.2 Surface polysacchar	2.3	10.7	3.8E-25	1.1E-23	M1	
STY0317	hypothetical protein	4.I Pathogenesis	2.2	8.1	3.5E-20	6.9E-19	M1	
STY1982	conserved hypothetical protein	5.H Hypothetical protein	2.2	6.5	3.4E-14	4.0E-13	M1	
STY3809	6-phosphofructokinase	1.B.1 Glycolysis	2.2	7.4	1.5E-17	2.3E-16	M1	D1M3
STY0755	hypothetical protein	5.I Unknown	2.2	5.4	1.4E-09	9.4E-09	M1	M3
nrdH	putative glutaredoxin	1.G.10 Thioredoxin	2.2	6.5	2.0E-13	2.1E-12	M1	
stbA	probable fimbrial protein	3.C.3 Surface structure	2.1	6.3	6.1E-13	6.3E-12	M1	
alID	ureidoglycolate dehydrogenase	1.A.1 Degradation of carbohydrate	2.1	6.6	1.5E-13	1.7E-12	M1	
STY2290	6-phosphogluconate dehydrogenase, decarboxylating	1.B.5 Pentose phosphate	2.1	9.3	8.3E-21	1.7E-19	M1	D1
STY4900	conserved hypothetical regulatory protein	2. Broad regulatory function	2.1	5.7	4.3E-10	3.1E-09	M1	
pduK	putative propanediol utilization protein PduK	1.A.1 Degradation of carbohydrate	2.1	7.1	4.6E-15	6.1E-14	M1	
yjga	conserved hypothetical protein	5.H Hypothetical protein	2.1	5.9	1.3E-10	9.7E-10	M1	D1M3

STY1756	conserved hypothetical protein	5.H Hypothetical protein	2.0	6.5	1.5E-12	1.5E-11	M1	
STY0185	PdxA-like protein	5.I Unknown	2.0	6.8	1.6E-13	1.7E-12	M1	
STY1169	putative transporter	4.A Transport/binding proteins	2.0	5.4	1.9E-08	1.1E-07	M1	
STY2088	conserved hypothetical protein	5.H Hypothetical protein	2.0	6.4	5.6E-12	5.1E-11	M1	
crcB	putative membrane protein	3.C.1 Membranes lipoprotein	2.0	5.8	5.5E-10	3.9E-09	M1	D1M3
STY1776	50S ribosomal subunit protein L35	3.A.2 Ribosomal protein synthesis, modification	10.6	5.8	5.3E-35	4.7E-34	M3	D1
STY3524	30S ribosomal subunit protein S9	3.A.2 Ribosomal protein synthesis, modification	10.3	5.5	8.1E-29	5.0E-28	M3	
STY3499	RNA polymerase sigma-54 factor (sigma-N)	2. Broad regulatory function	8.7	4.1	7.3E-13	2.1E-12	M3	D1M1
STY3464	30S ribosomal subunit protein S15	3.A.2 Ribosomal protein synthesis, modification	8.1	3.5	1.2E-08	2.7E-08	M3	
STY1498	haemolysin HlyE	4.H Cell Killing	8.0	3.4	5.9E-08	1.3E-07	M3	
STY2668	pseudogene	4.A.3 Transport Carbohydres, organic acids and alcohols	7.7	3.1	1.6E-06	3.1E-06	M3	D1
STY2860	50S ribosomal subunit protein L19	3.A.2 Ribosomal protein synthesis, modification	7.4	6.0	6.9E-36	6.4E-35	M3	
STY0954	transport ATP-binding protein CydC	4.A Transport/binding proteins	7.4	2.9	2.5E-06	4.8E-06	M3	
tufB	elongation factor Tu	3.A.8 Protein translation and modification	6.6	9.1	1.4E-90	2.4E-88	M3	D1D8M1
STY3466	ribosome-binding factor A (P15B protein)	3.A.8 Protein translation and modification	6.4	6.5	1.0E-42	1.4E-41	M3	
STY1357	peptide transport system permease protein SapC	4.A.6 Transport Other	6.4	6.0	3.7E-34	3.1E-33	M3	D1M1
trkA	potassium transport protein	4.A.2 Transport Cations	6.4	7.7	2.0E-63	1.1E-61	M3	D1
STY0786	cytochrome d ubiquinol oxidase subunit I	1.B.7.c Electron Transport	6.4	5.0	2.5E-21	1.0E-20	M3	
STY1299	DNA-binding protein (histone-like protein Hlp-II)	2. Broad regulatory function	6.1	6.6	1.2E-43	1.7E-42	M3	
STY1278	isocitrate dehydrogenase	1.B.3 Tricarboxylic acid cycle	6.1	4.8	3.6E-18	1.4E-17	M3	
STY1744	pyruvate kinase	1.B.1 Glycolysis	5.9	7.0	3.2E-48	6.5E-47	M3	D1
STY0516	haemolysin expression modulating protein	2. Broad regulatory function	5.9	8.5	7.5E-74	7.2E-72	M3	D1M1
STY3091	possible secreted protein	3.C.1 Membranes lipoprotein	5.8	4.5	2.5E-15	8.3E-15	M3	
STY2751	GMP synthase (glutamine-hydrolyzing)	1.F.1 Purine ribonucleotide biosynthesis	5.7	8.4	4.2E-70	3.4E-68	M3	D1M1
STY1356	peptide transport system ATP-binding protein SapD	4.A.6 Transport Other	5.5	5.6	4.5E-27	2.5E-26	M3	
STY1230	50S ribosomal protein L32	3.A.2 Ribosomal protein synthesis, modification	5.3	5.9	6.6E-29	4.1E-28	M3	M1
STY2568	phosphate acetyltransferase	1.A.1 Degradation of carbohydrate	5.3	4.9	6.7E-19	2.6E-18	M3	D1
STY3740	pantothenate kinase	1.G.5 Pantothenate	5.3	6.2	4.9E-33	3.8E-32	M3	
STY1368	peptide transport system permease protein SapB	4.A.6 Transport Other	5.3	7.1	1.9E-46	3.4E-45	M3	M1
STY4070	3-deoxy-D-manno-octulosonic-acid transferase	3.C.2 Surface polysacchar	5.2	5.4	4.8E-23	2.2E-22	M3	
STY0103	bis(5'-nucleosyl)-tetrakisphosphatase	1.F.4 Salvage of nucleosides and nucleotides	5.1	6.8	2.5E-41	3.1E-40	M3	D1M1
STY2106	crossover junction endodeoxyribonuclease	3.A.7 DNA replication, repair, restriction, modification	5.1	7.2	9.1E-47	1.7E-45	M3	
STY3914	ATP synthase epsilon subunit	1.A Degradation	5.1	5.3	4.9E-21	2.1E-20	M3	D1D8
waaQ	lipopolysaccharide core biosynthesis protein	3.C.2 Surface polysacchar	5.1	9.0	1.3E-70	1.1E-68	M3	D1
trkH	trk system potassium uptake protein	1.C.5 Sulphur Metabolism	4.9	9.6	2.0E-75	2.1E-73	M3	D1D8
STY0181	aconitate hydratase 2 (citrate hydro-lyase 2)	1.B.3 Tricarboxylic acid cycle	4.9	8.1	3.8E-57	1.4E-55	M3	D1

STY3809	6-phosphofructokinase	1.B.1 Glycolysis	4.8	9.8	1.4E-74	1.4E-72	M3	D1M1
STY0981	30S ribosomal protein S1	3.A.2 Ribosomal protein synthesis, modification	4.8	4.5	1.6E-13	4.6E-13	M3	
STY3904	glucose inhibited division protein	3.A.7 DNA replication, repair, restriction, modification	4.6	5.9	2.6E-27	1.5E-26	M3	D1M1
STY4285	conserved hypothetical protein	5.H Hypothetical protein	4.6	4.3	1.7E-11	4.7E-11	M3	M1
STY0386	hypothetical protein	5.I Unknown	4.5	4.8	1.5E-15	5.0E-15	M3	
hemK	HemK protein, putative protoporphyrinogen oxidase	5.I Unknown	4.5	5.8	1.3E-25	6.8E-25	M3	M1
STY1251	NADH dehydrogenase	1.B.7.a Aerobic Respiration	4.4	7.6	1.1E-46	2.1E-45	M3	D1M1
STY2586	putative decarboxylase	5.I Unknown	4.3	4.0	1.5E-09	3.6E-09	M3	M1
STY1355	peptide transport system ATP-binding protein SapF	4.A.6 Transport Other	4.2	7.8	3.4E-45	5.6E-44	M3	
STY0012	DnaK protein (heat shock protein 70)	4.B Chaperones	4.2	5.7	3.6E-23	1.6E-22	M3	D1
STY2836	ATP-dependent RNA helicase SrmB	3.A.9 RNA synthesis, modification	4.2	9.0	1.8E-56	6.6E-55	M3	D1D8
spiA	putative outer membrane secretory protein	4.I Pathogenesis	4.1	4.8	6.3E-14	1.9E-13	M3	D1
trpS	tryptophanyl-tRNA synthetase	3.A.5 Aminoacyl tRNA synthesis, tRNA modification	4.1	7.3	1.9E-39	2.2E-38	M3	D1D8M1
STY0956	thioredoxin reductase	1.F.3 2'-deoxyribonucleotide metabolism	4.0	6.9	7.4E-34	6.0E-33	M3	
STY0603	pseudogene	5.A Laterally acquired elements	3.9	6.7	5.8E-31	4.1E-30	M3	
yjga	conserved hypothetical protein	5.H Hypothetical protein	3.9	7.5	9.0E-39	1.0E-37	M3	D1M1
STY1771	integration host factor alpha-subunit	3.A.7 DNA replication, repair, restriction, modification	3.9	9.0	3.2E-51	8.1E-50	M3	
STY1893	putative bacteriophage protein	5.A Laterally acquired elements	3.8	4.2	1.2E-09	3.0E-09	M3	
STY4214	putative low-affinity inorganic phosphate transporter	4.A.5 Transport Anions	3.8	7.8	2.0E-41	2.6E-40	M3	D1
STY0970	hypothetical protein	5.I Unknown	3.8	6.7	1.3E-30	8.7E-30	M3	D1M1
STY3937	thiophene and furan oxidation protein	4.G Detoxification	3.7	5.8	2.2E-21	9.6E-21	M3	D1
STY1226	ribonuclease E	5.A Laterally acquired elements	3.7	7.2	9.8E-35	8.5E-34	M3	
STY2145	tyrosine-specific transport protein	4.A.1 Transport amino acid and amines	3.7	8.3	1.9E-43	2.7E-42	M3	D1D8
STY3886	molybdopterin-guanine dinucleotide biosynthesis protein A	1.G.4 Molybdopterin	3.7	8.5	1.5E-44	2.4E-43	M3	
STY4246	hypothetical protein	5.I Unknown	3.7	5.9	5.6E-22	2.5E-21	M3	M1
STY3463	polynucleotide phosphorylase	3.A.9 RNA synthesis, modification	3.6	11.5	1.1E-55	3.8E-54	M3	D1D8M1
STY1247	putative lipoprotein	3.C.1 Membranes lipoprotein	3.6	6.1	8.1E-24	3.9E-23	M3	
STY3975	conserved hypothetical protein	5.H Hypothetical protein	3.6	8.6	7.5E-44	1.1E-42	M3	
STY2078	hypothetical protein	5.I Unknown	3.6	5.9	1.4E-20	5.8E-20	M3	
STY1416	conserved hypothetical protein	5.H Hypothetical protein	3.5	7.1	4.2E-31	3.0E-30	M3	
STY1415	putative multi-drug transporter	5.D Drug/Analogue sensitivity	3.4	6.9	2.1E-28	1.3E-27	M3	
STY1038	putative bacteriophage protein	5.A Laterally acquired elements	3.4	5.9	6.1E-20	2.5E-19	M3	M1
STY1849	conserved hypothetical protein	5.H Hypothetical protein	3.4	7.5	8.9E-33	6.8E-32	M3	
STY2542	conserved hypothetical protein	5.H Hypothetical protein	3.4	8.0	3.2E-36	3.0E-35	M3	
STY0096	putative membrane protein	3.C.1 Membranes lipoprotein	3.3	7.4	3.0E-31	2.1E-30	M3	
STY1598	hypothetical protein	5.I Unknown	3.3	7.7	2.2E-33	1.7E-32	M3	



ompC	outer membrane protein C	3.C Cell envelope	3.3	7.6	8.4E-33	6.5E-32	M3	
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## Appendix B Papers published on aspects of this thesis

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### The Control of Typhoid Fever in Vietnam

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**Abstract.** Typhoid fever, caused by *Salmonella enterica* serovar Typhi (S. Typhi), is a diminishing public health problem in Vietnam, and this process may represent a prototype for typhoid elimination in Asia. Here, we review typhoid epidemiology in Vietnam over 20 years and assess the potential drivers associated with typhoid reduction. In the 1990s, multidrug resistant S. Typhi were highly prevalent in a sentinel hospital in southern Vietnam. A national typhoid incidence rate of 14.7/100,000 population per year was estimated around the new millennium. The Vietnamese government recognized the public health issue of typhoid in the 1990s and initiated vaccine campaigns to protect the most vulnerable members of the population. At their peak, these campaigns immunized approximately 1,200,000 children in 35 provinces. Concurrently, Vietnam experienced unprecedented economic development from 1998 to 2014, with the gross national income per capita increasing from \$360 to \$1,890 over this period. More recent typhoid incidence data are not available, but surveillance suggests that the current disease burden is negligible. This trajectory can be considered a major public health success. However, a paucity of systematic data makes it difficult to disaggregate the roles of immunization and water, sanitation, and hygiene (WASH) interventions in typhoid reduction in Vietnam. Given the limitations of typhoid vaccines, we surmise the practical elimination of typhoid was largely driven by economic development and improvement in general population living standards. Better designed WASH intervention studies with clinical endpoints and systematic incidence data are essential to glean a greater understanding of contextual factors that impact typhoid incidence reduction.

#### INTRODUCTION

Typhoid fever, the disease caused by *Salmonella enterica* serovar Typhi (S. Typhi), is a diminishing public health problem in Vietnam.<sup>1</sup> However, the disease remains an ongoing public health issue in other parts of South and Southeast Asia,<sup>2–4</sup> and an enhanced understanding of disease estimates and the influence of antimicrobial resistance (AMR) on disease presentation is needed to better control this disease across the region. Furthermore, insights into the trends of typhoid and factors that directly impinge on disease incidence are important for allocating resources for reducing the burden of disease.<sup>5</sup> Currently, Vietnam represents an exemplar Asian country that has all but eliminated this once common infection, and there is much to be learnt from the reduction of typhoid in Vietnam. However, how reduction in typhoid was precisely achieved is unclear, and providing a roadmap for typhoid reduction in similar settings is largely dependent on good historical quantitative data.

**Typhoid fever incidence in Vietnam.** Typhoid fever has likely been endemic in Vietnam for some time, although historical incidence data for this common cause of febrile disease from across Southeast Asia before the reunification of Vietnam in 1975 is scarce. Notified typhoid fever cases reported to the pre-reunification government of South Vietnam showed a generally increasing trend from 2.05 cases per 100,000 people annually in 1957 to 10.02 cases per 100,000 people annually in 1966 (Figure 1).<sup>6</sup> Health care provisions, and water and sanitation infrastructure in South Vietnam during this time period were generally poor, which likely contributed to the increasing rates of typhoid fever and other infectious disease during this period, which were frequently observed in military personnel

returning back to the United States.<sup>7</sup> By contrast, a report suggests that the pre-reunification government in North Vietnam prioritized health care access and began mass vaccination campaigns against typhoid fever and other communicable diseases as early as 1954, although reliable incidence data from North Vietnam during this period are not available.<sup>8</sup>

The best and most accurate recent estimates of typhoid incidence in Vietnam were calculated during the International Vaccine Institute's Diseases of the Most Impoverished (DOMI) program, which was conducted between 1999 and 2003.<sup>9</sup> The annual incidence of typhoid fever in Hue, in central Vietnam, in 2002–2004 was estimated to be 21.3/100,000 person years and 24.2/100,000 person years in children aged 5–15 in years (Figure 1). This program went on to conduct various epidemiological investigations and vaccine studies in the same location.<sup>10,11</sup> In addition, the National Institute of Epidemiology (NIHE) in Hanoi conducted further nationwide surveillance around the same period of time as the DOMI study. The average number of typhoid cases in Vietnam across the country in all ages was estimated to be 11,696, corresponding with an average national incidence rate of 14.7/100,000 population per year.<sup>12</sup> During this period (1999–2003) two of the 63 provinces of Vietnam (Soc Trang in the south and Dien Bien in the north) were estimated to have particularly high incidences (> 100/100,000 population per year) (Figure 2); a further 18 were estimated to have a medium incidence (> 10 < 100/100,000 population per year). The propensity of the disease was understood to arise in children, with an estimated incidence of 36.6/100,000 population aged < 15 years per year.<sup>12</sup> Lastly, in 1998, Lin and others.<sup>13</sup> estimated a population incidence of 198/100,000 population in the Dong Thap province in the Mekong Delta, equating with a crude incidence of 930/100,000 people (Figure 1).

**Typhoid trends in Ho Chi Minh City (HCMC).** Routine blood culture data from the Hospital for Tropical Diseases

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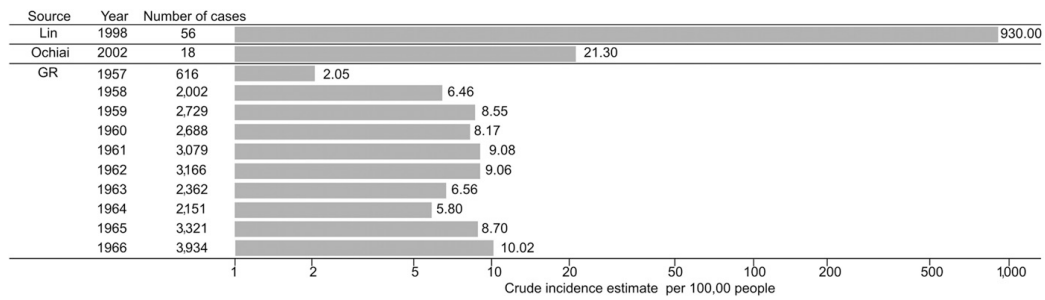


FIGURE 1. Historic crude estimates of typhoid fever incidence in Vietnam. Histogram showing the estimated crude incidences (on a log scale) of typhoid fever in Vietnam from government records (GR) and subnational incidence estimates available from Ochiai et al.<sup>9</sup> (aggregated estimated from 2002 to 2004, with 2003 as the midpoint from Hue province in people aged 5–18 years) and Lin et al.<sup>13</sup> (aggregated estimated from 1997 to 2000, with 1998 as the midpoint from Dong Thap province).

(HTD) in HCMC in the south of Vietnam between 1994 and 2015 highlights a major reduction in the prevalence (and absolute number) of positive blood cultures for *S. Typhi* over time (Figure 3).<sup>1</sup> Hospital for Tropical Diseases is a sentinel infectious disease hospital that serves as a primary and secondary facility for the surrounding local population in HCMC and a tertiary referral center for 17 provinces in the south of the country, and, therefore, has a catchment population of approximately 40 million people. The highest rates of positive blood cultures for *S. Typhi* at HTD were recorded in 1995 and

1998, when the proportion of positive blood cultures for *S. Typhi* was 14.5% and 12.8% (of all blood cultures taken), respectively. In the late 1990s, this figure began to show an annual decline; 5% *S. Typhi* blood culture positivity rate of all blood cultures taken in 1999. After the turn of the millennium, the number of culture positive cases of typhoid fever at HTD continued to decrease annually, with the prevalence of *S. Typhi*-positive blood cultures not rising higher than 1% from 2005 onward. Therefore, in the absence of contemporary (and accurate incidence) data, if we extrapolate these trends we

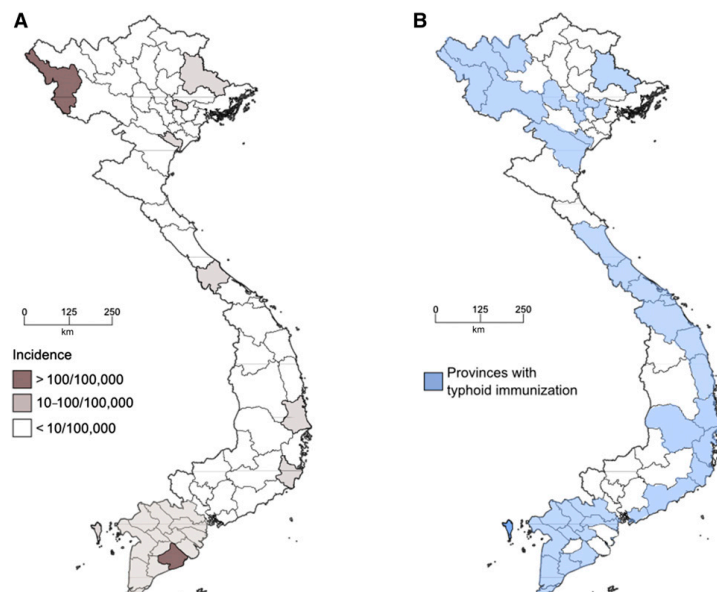


FIGURE 2. Map of Vietnam showing estimated disease incidences and provinces implemented Vi immunization. (A) North orientated map of Vietnam showing the estimated incidence of typhoid fever in Vietnam from government data between 1999 and 2003. Provinces with high, medium, and low incidence are highlighted by shading (see key). (B) North orientated map of Vietnam showing the 35 provinces in Vietnam in 2005 that were incorporated into the national typhoid Vi immunization campaign; blue shading (see key). Maps are reproduced from Cuong N. Typhoid Vaccine Used in Vietnam and its Impact. In: Consultation on Typhoid Vaccine Introduction and Typhoid Surveillance.<sup>12</sup>

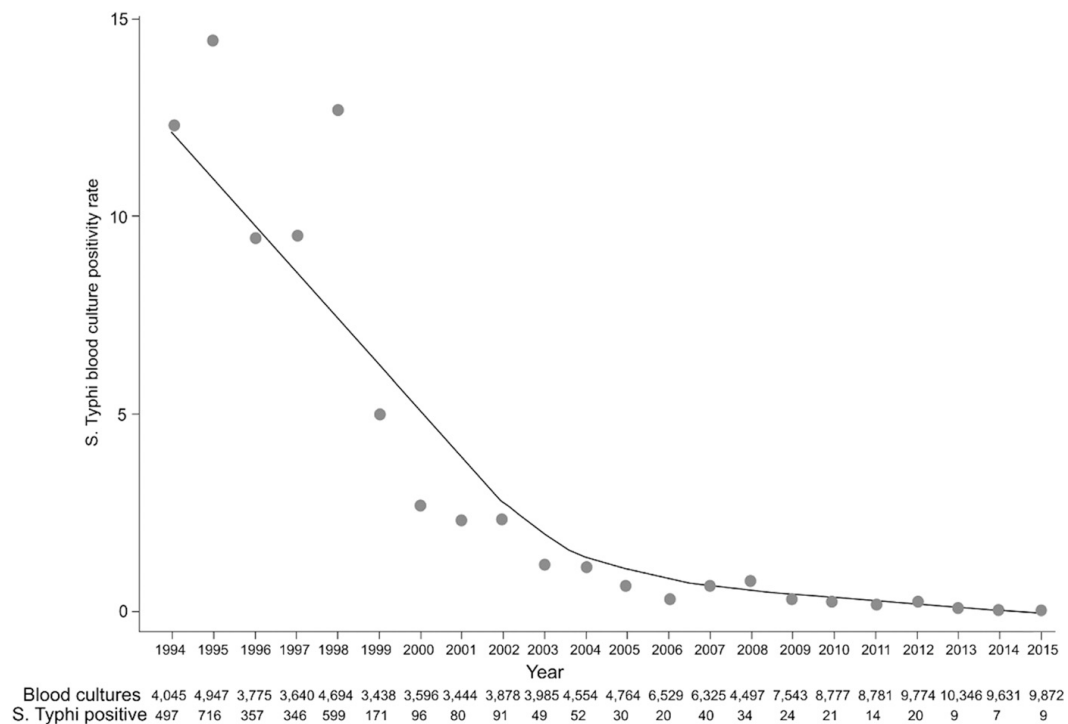


FIGURE 3. The decline in *Salmonella* Typhi-positive blood cultures in a sentinel infectious disease hospital in Ho Chi Minh City (HCMC). Plot showing the proportion of total blood cultures taken from which *Salmonella* Typhi was isolated between 1993 and 2015 at the Hospital for Tropical Diseases in HCMC, with a locally weighted scatterplot smoothing curve. The total number of blood cultures taken and the number from which *Salmonella* Typhi were isolated are shown at the base of the figure.

can surmise that presently the incidence of enteric fever in Vietnam is probably exceptionally low ( $< 10/100,000$  population per year), and there has been a remarkable and sustained decline in the prevalence of *S. Typhi*-positive blood cultures in HTD and other health care facilities across the country.

In a pattern similar to those observed in parts of sub-Saharan Africa (but not in the same magnitude), there has been a replacement of “classical” community-acquired pathogens in bloodstream infections (such as *S. Typhi*) with those more commonly associated with HIV infection and the current international epidemic of AMR bacteria.<sup>14</sup> Specifically, assorted fungal pathogens, multidrug-resistant (MDR) non-*Salmonella* Gram-negative bacteria, and non-typhoidal *Salmonella* now dominate the bloodstream infection landscape in Vietnam.<sup>1,15,16</sup> Paratyphoid fever, which is associated with the various pathovars of *Salmonella* Paratyphi (*S. Paratyphi* A, B, and C), has been reported to be increasing in prevalence in parts of Asia.<sup>17</sup> This surge is specifically associated with *S. Paratyphi* A; however, the isolation of this organism is rare in Vietnam (and across Southeast Asia) and is generally limited to extended sporadic outbreaks, as recently observed in neighboring Cambodia.<sup>18</sup> A study conducted at HTD cultured less than 7 *S. Paratyphi* A isolates per year between 1998 and 2008, this subsequently declined to zero from 2008.<sup>1</sup>

However, in 1990, Global Burden of Disease estimated incidence of paratyphoid to be  $81/100,000$  population; this was estimated to be  $40/100,000$  population in 2016.<sup>19</sup>

**Antimicrobial susceptibility.** Traditionally, Vietnam has been a global hotspot for multi-drug resistant (MDR) *S. Typhi*, which is defined as resistance against the first-line antimicrobials, ampicillin, chloramphenicol, and trimethoprim-sulphate.<sup>20</sup> These latter day first-line regimes were commonly prescribed in the community and in health care settings for the treatment of typhoid fever, and many non-specific febrile diseases, in Vietnam in the 1980s and early 1990s. The first notable spike of MDR *S. Typhi* in Vietnam arose in the early 1990s, correlating with a major peak in *S. Typhi*-positive blood cultures at HTD in HCMC.<sup>21</sup> This MDR phenotype in *S. Typhi* was associated with an *incH1* plasmid backbone,<sup>22</sup> which has been consistently identified within *S. Typhi* isolated in Vietnam and coupled with organisms belonging to a specific phylogenetic group known as haplotype 58,<sup>23</sup> now designated genotype 4.3.1.<sup>24</sup> These organisms, with this same MDR phenotype, were described as still circulating in high numbers in the Mekong Delta region, some 150 km away from HCMC, in 2004 and 2005.<sup>25</sup>

*Salmonella enterica* serovar Typhi belonging to genotype 4.3.1 are also commonly associated with a mutation (S83F) in

the DNA gyrase gene *gyrA*, catalyzing resistance against naladixic acid and reduced susceptibility against the second-generation fluoroquinolones, ciprofloxacin, and ofloxacin.<sup>26,27</sup> Variants with this specific *gyrA* mutation began to emerge in Vietnam in the early 1990s,<sup>28</sup> shortly after the introduction of quinolones for the treatment of non-specific febrile diseases when the first-line treatments became less effective at inducing defervescence. The secondary peak in *S. Typhi* cases in routine blood culture data from HTD was associated with the emergence of organisms exhibiting resistance against quinolones and reduced susceptibility against fluoroquinolones.<sup>1,29</sup> These organisms, specifically genotype 4.3.1 *S. Typhi* with an S83F mutation in *gyrA*, have since become the most prevalent variant in Vietnam,<sup>25</sup> reflecting the pattern of the molecular epidemiology of *S. Typhi* across much of Asia.<sup>27</sup> Current information regarding the AMR profiles of the extant *S. Typhi* population in Vietnam are limited, but our unpublished data suggests that MDR strains have all but disappeared in the southern part of the country and *S. Typhi* with an S83F and reduced susceptibility to fluoroquinolones continue to circulate. Notably, despite the sustained use and availability of fluoroquinolones in Vietnam for various bacterial infections, ciprofloxacin- and ofloxacin-resistant *S. Typhi* has yet to emerge, although it has been observed elsewhere in Asia.<sup>30</sup>

**Vaccination campaigns.** The Vietnamese government recognized the public health issue of typhoid fever in the 1990s and initiated several vaccine campaigns with internationally manufactured vaccines in an attempt to protect the most vulnerable groups within the population, most commonly children.<sup>31</sup> Furthermore, NIHE in Hanoi was a pioneer in instigating locally manufactured Vi Polysaccharide vaccine and distributing it as a control measure through the public health network. However, despite a Vi conjugate vaccine and a new oral attenuated typhoid vaccine being trialed in Vietnam for the first time, these were never introduced as public health interventions.<sup>32,33</sup> All the national typhoid Vi Polysaccharide vaccine programs conducted between 1997 and 2012 were executed as school-based campaigns, immunizing children between the ages of 3 and 10 years. Between 1997 and 2003, the Vietnamese government immunized more than 4,000,000 children aged 3–5 years with TYPHIM Vi polysaccharide, manufactured by Aventis Pasteur. After manufacturing their own Vi polysaccharide vaccine through the Institute of Vaccines and Medical Biologicals/Da Lat Pasteur Vaccine Company, the Vietnamese public health system administered more than 2,000,000 additional doses to children aged 5–10 years (2004–2010), and latterly children aged 3–5 years (2011–2012). At the peak of these Vi vaccine campaigns in 2005 (1,200,000 doses), children were being immunized in 35 different provinces across the country (Figure 2).<sup>12</sup> The coverage rate of these campaigns were high, with > 90% of the target population receiving a Vi vaccine between 1999 and 2010. Population based data assessing the direct effect (i.e., without additional sanitation covariates) of these immunization programs on typhoid incidence are unavailable, but there was a substantial decrease in the incidence of typhoid fever across the country between 1997 and 2007, most notably in the northwest of the country and the Mekong River Delta in the South, which were covered extensively by the immunization program (Figure 2).<sup>12</sup>

**Contextual factors that may have influenced typhoid fever incidence.** Being of low socioeconomic status is a

major risk factor for contracting typhoid fever.<sup>34</sup> Vietnam has been through an unprecedented period of economic development since the mid-1990s, which has had a substantial knock-on effect on the reduction of poverty and poverty-associated communicable diseases.<sup>35</sup> Between 1998 and 2014 Vietnam's gross domestic product increased from \$27 billion to \$86 billion, and the gross national income per capita increased from \$360 to \$1,890 over the same time period.<sup>36</sup> These figures correlate with a reduction in national poverty (poverty headcount ratio at \$1.90 a day declining from 34.8% in 1998 to 3.2% in 2012) (Figure 4) and a waning in typhoid fever incidence, but overlap with the period of the national immunization campaigns. In addition, multiple contextual factors are considered to have had an effect on the incidence of typhoid fever. Typhoid fever is associated with poor water quality, and evidence of the organism in water supplies can be measured using molecular methods.<sup>37</sup> Consequently, water, sanitation, and hygiene (WASH) conditions are one of the major factors in assessing disease control. Sub-national data on water and sanitation extracted from Multiple Indicator Cluster Surveys (MICS) in Vietnam show that the fraction of the population in the southeast of Vietnam with access to improved sanitation facilities (means of excreta disposal that decrease human contact with feces) increased from only 42% in 1995 to 93.6% in 2014.<sup>38</sup> Furthermore, the proportion of the southeastern population of Vietnam with improved water sources (as per MICS standards; water piped into homes or yard, public taps, standpipes, protected wells, or springs) rose from 93.6% in 2006 to a peak of 98.4% in 2011.<sup>38</sup> Correspondingly, data from the World Bank shows that there was a steady increase in the proportion of the Vietnamese population with access to improved sources of drinking water between 1998 (74.5% of the population) and 2014 (96.4% of the population).<sup>39</sup> Similarly, there was an increase in the proportion of the population with access to improved sanitation facilities, including flush and slab latrines, over the same period; 49.5% of the population in 1998 to 76.3% of the population in 2012 (urban/rural data shown in Figure 4).<sup>39</sup>

**Knowledge gaps.** All available data suggest that the trend of typhoid fever incidence began to exhibit a steep decline in Vietnam from 1999 onward. Therefore, the outstanding questions regarding the dramatic reduction in typhoid fever in Vietnam are: to what extent did immunization play a role in reducing typhoid fever? and how much influence did economic growth have on improving living standards to reduce all waterborne diseases, including typhoid fever? Given the paucity of data regarding the longitudinal incidences of typhoid fever (and other waterborne diseases) in Vietnam, disaggregating the independent effect of these differing approaches is a major challenge. Furthermore, dissimilar demographics, disease epidemiology, and disease incidence across the provinces of Vietnam make it impractical to assess the overall impact of a specific intervention in any given location. The data that are available are inconclusive and limited by disease time trends from a single tertiary referral hospital in HCMC. As a result, these data do not provide insight into regional differences in typhoid fever reduction across Vietnam or characterize the current national burden. Furthermore, there are no systematic data regarding intestinal perforation (although this has been described in the southern provinces of Vietnam<sup>40</sup>), other

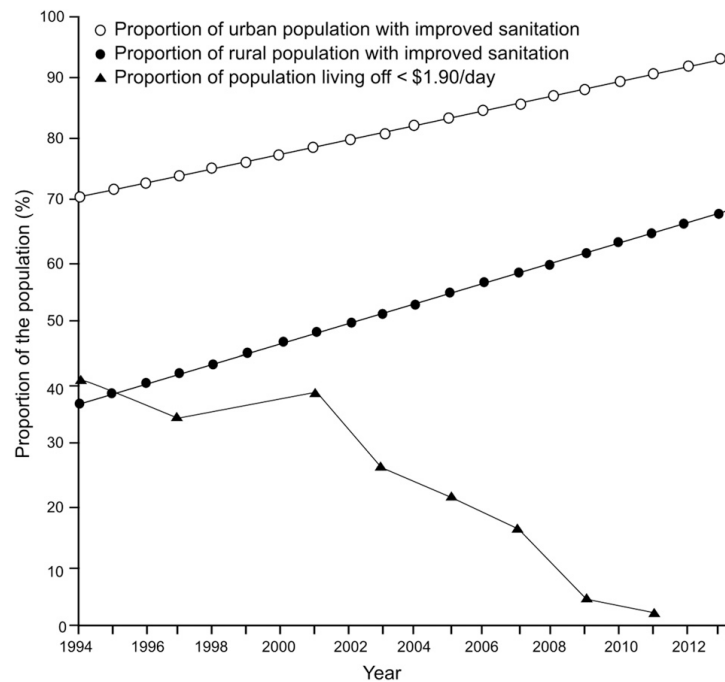


FIGURE 4. The reduction of poverty and improvements in sanitation in Vietnam. Plots showing the proportion of the Vietnamese population living on < \$1.90 a day (black triangles), the proportion of the Vietnamese rural population with improved sanitation (black circles), and the proportion of the Vietnamese urban population with improved sanitation (white circles) from 1994 to 2013.

severe disease presentations,<sup>41</sup> or typhoid-associated mortality. As a result, it is impossible to directly assess any trends associated with these severe outcomes.

Regardless of the precise role of any single intervention, the reduction and virtual elimination of typhoid fever in Vietnam, largely driven by strong political will, as evidenced by sanitation improvements, better health care, and immunization campaigns, should be considered a major public health success. However, this success story does not quite replicate into a blueprint in how typhoid fever should be controlled across Asia, that is, what worked in Vietnam may not work in precisely the same way in other locations. It is likely that the various vaccination campaigns that have taken place in Vietnam over the last 15 years have contributed to the observed decreasing trend in typhoid fever, but their effect is difficult to assess. However, the immunization campaigns were conducted in children in selected provinces, and these individuals received a single dose of the Vi polysaccharide vaccine. This vaccine only provides limited efficacy in the first year after immunization and there is a rapid decline in antibody titer 2 years after receiving the vaccine,<sup>42,43</sup> herd protection remains variable. Therefore, we conclude that economic development, improved access to clean water supplies, better sanitation, and a reduction in poverty probably played the greatest combined role in reducing the incidence of typhoid in Vietnam. Quantifying the precise contribution of these in the reduction of typhoid incidence is again problematic, and there are multiple additional

factors that should also be considered that may confound these interactions. It is, however, worth stating that *S. Typhi* appears to be profoundly sensitive to improvements in sanitation, which reduces human exposure to the organism, thus, lessening disease incidence and person-to-person transmission.

#### CONCLUSION

The reduction of typhoid fever in Vietnam has been remarkable, and has been largely driven by economic development and improved living standards for the population. Immunization has probably had some impact on disease reduction, but the use of an imperfect vaccine may only provide limited respite in disease transmission without required improvements in WASH. Better designed WASH intervention studies with disease endpoints and systematic incidence data are required to glean a greater understanding of the precise contextual factors that impact on typhoid fever incidence.

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# REFERENCES

1. Nga TVT et al., 2012. The decline of typhoid and the rise of non-typhoid salmonellae and fungal infections in a changing HIV landscape: bloodstream infection trends over 15 years in southern Vietnam. *Trans R Soc Trop Med Hyg* 106: 26–34.
2. Pham Thanh D et al., 2016. The molecular and spatial epidemiology of typhoid fever in rural Cambodia. *PLoS Negl Trop Dis* 10: e0004785.
3. Baker S et al., 2011. Combined high-resolution genotyping and geospatial analysis reveals modes of endemic urban typhoid fever transmission. *Open Biol* 1: 110008.
4. Hendriksen RS et al., 2015. Genomic dissection of travel-associated extended-spectrum-beta-lactamase-producing *Salmonella enterica* serovar typhi isolates originating from the Philippines: a one-off occurrence or a threat to effective treatment of typhoid fever? *J Clin Microbiol* 53: 677–680.
5. Baker S, Hombach J, Marks F, 2016. What have we learned from the typhoid fever surveillance in Africa program? *Clin Infect Dis* 62 (Suppl 1): S1–S3.
6. World Health Organization, 1968. *Epidemiological Situation in Viet-Nam*. Twenty-first World Health Assembly Agenda, May 6–24, 1968, Geneva, Switzerland.
7. Greenberg JH, 1969. Public health problems relating to the Vietnam returnee. *JAMA* 207: 697–702.
8. Levy BS, Sidel V, 2008. *War and Public Health*, 1st edition. New York, NY: Oxford University Press.
9. Ochiai RL et al.; Domi Typhoid Study Group, 2008. A study of typhoid fever in five Asian countries: disease burden and implications for controls. *Bull World Health Organ* 86: 260–268.
10. Ali M, Thiem VD, Park JK, Ochiai RL, Canh DG, Danovaro-Holliday MC, Kaljee LM, Clemens JD, Acosta CJ, 2007. Geographic analysis of vaccine uptake in a cluster-randomized controlled trial in Hue, Vietnam. *Health Place* 13: 577–587.
11. Cook J, Jeuland M, Whittington D, Poulos C, Clemens J, Sur D, Anh DD, Agtini M, Bhutta Z; DOMI Typhoid Economics Study Group, 2008. The cost-effectiveness of typhoid Vi vaccination programs: calculations for four urban sites in four Asian countries. *Vaccine* 26: 6305–6316.
12. Cuong N, 2011. *Typhoid Vaccine Used in Vietnam and its Impact*. Consultation on Typhoid Vaccine Introduction and Typhoid Surveillance. Available at: <https://www.sabin.org/sites/sabin.org/files/CUONG%20Typhoid%20Vaccine%20in%20Vietnam.pdf>. Accessed June 10, 2018.
13. Lin FY, Vo AH, Phan VB, Nguyen TT, Bryla D, Tran CT, Ha BK, Dang DT, Robbins JB, 2000. The epidemiology of typhoid fever in the Dong Thap Province, Mekong Delta region of Vietnam. *Am J Trop Med Hyg* 62: 644–648.
14. Gordon MA, Banda HT, Gondwe M, Gordon SB, Boeree MJ, Walsh AL, Corkill JE, Hart CA, Gilks CF, Molyneux ME, 2002. Non-typhoidal *Salmonella* bacteraemia among HIV- infected Malawian adults: high mortality and frequent recrudescence. *AIDS* 16: 1633–1641.
15. Le T et al., 2011. Epidemiology, seasonality, and predictors of outcome of AIDS-associated *Penicillium mameffei* infection in Ho Chi Minh City, Viet Nam. *Clin Infect Dis* 52: 945–952.
16. Phu Huong Lan N et al., 2016. Invasive non-typhoidal *Salmonella* infections in Asia: clinical observations, disease outcome and dominant serovars from an infectious disease hospital in Vietnam. *PLoS Negl Trop Dis* 10: e0004857.
17. Ochiai RL et al., 2005. *Salmonella* paratyphi A rates, Asia. *Emerg Infect Dis* 11: 1764–1766.
18. Vlieghe E, Phe T, De Smet B, Veng CH, Kham C, Sar D, van Griensven J, Lim K, Thai S, Jacobs J, 2013. Increase in *Salmonella enterica* serovar Paratyphi A infections in Phnom Penh, Cambodia, January 2011 to August 2013. *Euro Surveill* 18: pii: 20592.
19. Global Burden of Disease, 2018. *Global Burden of Disease Results Tool*. GBD Results Tool | GHDX. Available at: <http://ghdx.healthdata.org/gbd-results-tool>. Accessed May 1, 2018.
20. Chinh NT, Parry CM, Ly NT, Ha HD, Thong MX, Diep TS, Wain J, White NJ, Farrar JJ, 2000. A randomized controlled comparison of azithromycin and ofloxacin for treatment of multidrug-resistant or nalidixic acid-resistant enteric fever. *Antimicrob Agents Chemother* 44: 1855–1859.
21. Hoa NT, Diep TS, Wain J, Parry CM, Hien TT, Smith MD, Walsh AL, White NJ, 1998. Community-acquired septicemia in southern Viet Nam: the importance of multidrug-resistant *Salmonella typhi*. *Trans R Soc Trop Med Hyg* 92: 503–508.
22. Wain J et al., 2003. Molecular analysis of *incHI1* antimicrobial resistance plasmids from *Salmonella* serovar Typhi strains associated with typhoid fever. *Antimicrob Agents Chemother* 47: 2732–2739.
23. Holt KE et al., 2011. Emergence of a globally dominant *incHI1* plasmid type associated with multiple drug resistant typhoid. *PLoS Negl Trop Dis* 5: e1245.
24. Wong VK et al.; International Typhoid Consortium, 2016. An extended genotyping framework for *Salmonella enterica* serovar Typhi, the cause of human typhoid. *Nat Commun* 7: 12827.
25. Holt KE et al., 2011. Temporal fluctuation of multidrug resistant *Salmonella typhi* haplotypes in the Mekong River Delta region of Vietnam. *PLoS Negl Trop Dis* 5: e929.
26. Parry CM, Vinh H, Chinh NT, Wain J, Campbell JI, Hien TT, Farrar JJ, Baker S, 2011. The influence of reduced susceptibility to fluoroquinolones in *Salmonella enterica* serovar Typhi on the clinical response to ofloxacin therapy. *PLoS Negl Trop Dis* 5: e1163.
27. Wong VK et al., 2015. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of *Salmonella typhi* identifies inter- and intracontinental transmission events. *Nat Genet* 47: 632–639.
28. Wain J et al., 1997. Quinolone-resistant *Salmonella typhi* in Viet Nam: molecular basis of resistance and clinical response to treatment. *Clin Infect Dis* 25: 1404–1410.
29. Connerton P et al., 2000. Epidemic typhoid in Vietnam: molecular typing of multiple-antibiotic-resistant *Salmonella enterica* serotype typhi from four outbreaks. *J Clin Microbiol* 38: 895–897.
30. Pham Thanh D et al., 2016. A novel ciprofloxacin-resistant sub-clade of H58 *Salmonella typhi* is associated with fluoroquinolone treatment failure. *ELife* 5: e14003.
31. DeRoek D, Ochiai RL, Yang J, Anh DD, Alag V, Clemens JD, 2008. Typhoid vaccination: the Asian experience. *Expert Rev Vaccines* 7: 547–560.
32. Tran TH et al., 2010. A randomised trial evaluating the safety and immunogenicity of the novel single oral dose typhoid vaccine M01ZH09 in healthy Vietnamese children. *PLoS One* 5: e11778.
33. Lin FY et al., 2001. The efficacy of a *Salmonella typhi* Vi conjugate vaccine in two-to-five-year-old children. *N Engl J Med* 344: 1263–1269.
34. Karkey A et al., 2013. Differential epidemiology of *Salmonella* Typhi and Paratyphi A in Kathmandu, Nepal: a matched case control investigation in a highly endemic enteric fever setting. *PLoS Negl Trop Dis* 7: e2391.
35. Vandemoortele M, Bird K, Steer L, 2011. *Viet Nam's Progress on Economic Growth and Poverty Reduction: Impressive Improvements*. London. Available at: [http://www.developmentprogress.org/sites/developmentprogress.org/files/vietnam\\_report\\_-\\_master\\_0.pdf](http://www.developmentprogress.org/sites/developmentprogress.org/files/vietnam_report_-_master_0.pdf). Accessed June 10, 2018.
36. IndexMundi, 2016. *Facts About Vietnam*. Available at: <http://www.indexmundi.com/facts/vietnam>. Accessed January 13, 2017.

37. Karkey A et al., 2016. The ecological dynamics of fecal contamination and *Salmonella* Typhi and *Salmonella* Paratyphi a in municipal Kathmandu drinking water. *PLoS Negl Trop Dis* 10: e0004346.
38. UNICEF, 2015. *Multiple Indicator Cluster Survey (MICS)*. Statistics and Monitoring. Available at: [https://www.unicef.org/statistics/index\\_countrystats.html](https://www.unicef.org/statistics/index_countrystats.html). Accessed January 13, 2017.
39. The World Bank, 2016. *World Bank Open Data—Vietnam*. Available at: <http://data.worldbank.org/country/vietnam>. Accessed January 13, 2017.
40. Nguyen QC et al., 2004. A clinical, microbiological, and pathological study of intestinal perforation associated with typhoid fever. *Clin Infect Dis* 39: 61–67.
41. Parry CM, Thompson C, Vinh H, Chinh NT, Phuong le T, Ho VA, Hien TT, Wain J, Farrar JJ, Baker S, 2014. Risk factors for the development of severe typhoid fever in Vietnam. *BMC Infect Dis* 14: 73.
42. Khan MI, Soofi SB, Ochiai RL, Habib MA, Sahito SM, Nizami SQ, Acosta CJ, Clemens JD, Bhutta ZA; DOMI Typhoid Karachi Vi Effectiveness Study Group, 2012. Effectiveness of Vi capsular polysaccharide typhoid vaccine among children: a cluster randomized trial in Karachi, Pakistan. *Vaccine* 30: 5389–5395.
43. Sur D et al., 2009. A cluster-randomized effectiveness trial of Vi typhoid vaccine in India. *N Engl J Med* 361: 335–344.





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## An evaluation of purified *Salmonella* Typhi protein antigens for the serological diagnosis of acute typhoid fever



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## KEYWORDS

Typhoid fever;  
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*Salmonella* Typhi;  
Diagnostics;  
Bangladesh;  
Vi polysaccharide;  
IgM;  
Febrile disease

**Summary** *Objectives:* The diagnosis of typhoid fever is a challenge. Aiming to develop a typhoid diagnostic we measured antibody responses against *Salmonella* Typhi (*S. Typhi*) protein antigens and the Vi polysaccharide in a cohort of Bangladeshi febrile patients.

*Methods:* IgM against 12 purified antigens and the Vi polysaccharide was measured by ELISA in plasma from patients with confirmed typhoid fever ( $n = 32$ ), other confirmed infections ( $n = 17$ ), and healthy controls ( $n = 40$ ). ELISAs with the most specific antigens were performed on plasma from 243 patients with undiagnosed febrile disease.

*Results:* IgM against the *S. Typhi* protein antigens correlated with each other ( $\rho > 0.8$ ), but not against Vi ( $\rho < 0.6$ ). Typhoid patients exhibited higher IgM against 11/12 protein antigens and Vi than healthy controls and those with other infections. Vi, PiL, and CdtB exhibited the greatest sensitivity and specificity. Specificity and sensitivity was improved when Vi was combined with a protein antigen, generating sensitivities and specificities of 0.80 and  $>0.85$ , respectively. Applying a dynamic cut-off to patients with undiagnosed febrile disease suggested that 34–58% had an IgM response indicative of typhoid.

*Conclusions:* We evaluated the diagnostic potential of several *S. Typhi* antigens; our assays give good sensitivity and specificity, but require further assessment in differing patient populations.

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## Introduction

Enteric (typhoid) fever is a systemic infection caused by *Salmonella enterica* serovars Typhi (*S. Typhi*) and Paratyphi A (*S. Paratyphi* A).<sup>1,2</sup> There are an estimated 12 million cases of typhoid (*S. Typhi* only) worldwide annually leading to approximately 120,000 deaths.<sup>3,4</sup> The organisms are transmitted via the fecal-oral route and the disease remains common in low/middle income countries in South/Southeast Asia and sub-Saharan Africa.<sup>5</sup> Despite *S. Paratyphi* A being an emergent cause of enteric fever in parts of South and Southeast Asia,<sup>6</sup> *S. Typhi* remains the most commonly reported etiological agent of enteric fever in Asia and Africa.

Typhoid occurs only in humans, making it a disease that can technically be eradicated.<sup>7</sup> Indeed, typhoid has all but been eliminated from several countries in Southeast Asia where it was the most common cause of hospitalized febrile disease 20–30 years ago.<sup>8,9</sup> Elimination in these areas is generally attributed to extensive improvements in sanitation rather than widespread immunization schemes. The lack of data regarding the long-term impact of mass immunization for typhoid and the performance of licensed vaccines have hindered immunization as a sustainable typhoid control and elimination strategy. Future considerations for rational control measures for typhoid will rely on more accurately assessing disease burden, which requires a reliable diagnostic approach.<sup>10</sup>

All commonly used typhoid diagnostics perform poorly and are a roadblock for disease control efforts.<sup>11,12</sup>

Currently, the only reliable method for the identification of febrile individuals with typhoid is the culture of a causative organism from a biological specimen.<sup>12,13</sup> However, this procedure is restricted to laboratories with adequate equipment and microbiology training, and the method has a limited sensitivity due to low concentrations of organisms in the peripheral circulation.<sup>14–16</sup> Low bacterial loads have a similar impact on other methods that rely on detecting the presence of the infecting organisms, such as antigen detection or nucleic acid amplification. These methods are often reported to be highly sensitive, but have unrealistic performances; pre-treatment with antimicrobials is likely to compound this issue further.<sup>11,15</sup> New typhoid diagnostics are a necessity and various approaches have been evaluated, including measurement of innate immune responses,<sup>17,18</sup> antibody in lymphocyte supernatants,<sup>19,20</sup> and the identification of metabolomic signatures.<sup>21</sup> However, these advances are still restricted to research laboratories and are not yet ready to be developed into simple, rapid diagnostic tests (RDTs).

We previously exploited a protein microarray to identify a multitude of immunogenic *S. Typhi* protein antigens to which an antibody response was generated during the early stages of typhoid.<sup>22</sup> With the aim of validating antigens that could be used in a diagnostic assay we expressed and purified several of these potentially serodiagnostic *S. Typhi*

antigens and investigated their diagnostic performance in a cohort of febrile Bangladeshi patients.

## Materials and methods

### Ethical approval

The study was conducted according to the principles expressed in the Declaration of Helsinki. The Bangladesh National Research Ethical Committee (BMRC/NREC/2010-2013/1543), the Chittagong Medical College Hospital Ethical Committee, the Oxford Tropical Research Ethics Committee (OXTREC 53-09) gave ethical approval for the study. Informed written or thumbprint consent was taken from the subject, their parent or caretaker for all enrollees.

### Study site, population and study design

The site and recruitment for this study has been described previously.<sup>23</sup> Briefly, Chittagong Medical College Hospital is a 1000-bed hospital serving Chittagong and the surrounding province. Adults and children (>6 months) consecutively admitted from January to June 2012 to the adult and pediatric wards at CMCH with an axillary temperature of  $\geq 38^{\circ}\text{C}$  up to 48 h after admission and history of fever for <2 weeks were eligible for the study.

Here the gold standards for typhoid diagnosis were and blood culture and PCR amplification from blood using a previously described method.<sup>15</sup> Blood (5–12 mL for adults and 1–2 mL for children) was cultured using Bact/Alert-FA and PF blood culture bottles, bottles were incubated in the Bact/Alert automated system (Biomérieux, France) for five days. The patient demographics and diagnostic testing results for this study are reported elsewhere.<sup>23</sup> For the purposes of this investigation plasma samples from 40 healthy adult control subjects (hospital staff at

the study site), 32 cases of confirmed typhoid (16 cases confirmed by blood culture, 13 cases confirmed by PCR and three cases confirmed by both blood culture and PCR), 17 cases from patients with confirmed febrile diseases other than enteric fever (*Staphylococcus aureus* (2), *Streptococcus pneumoniae* (1), *Streptococcus acidominimus* (1), *Enterococcus gallinarum* (1), *Escherichia coli* (2), *Klebsiella pneumoniae* (1), *Enterobacter cloacae* (2), *Acinetobacter* spp. (1), *Burkholderia cepacia* (3), dengue (2), *R. typhi* (2) and *O. tsutsugamushi* (1)) and 243 febrile patients with undiagnosed febrile disease were subjected to serological assays.

### PCR amplification, gene expression and protein purification

We selected 18 *S. Typhi* antigens that gave a differential serodiagnostic signal using protein microarray screening for further expression and purification (Table S1). The coding sequences of the selected genes, excluding transmembrane domains were PCR amplified from CT18 genomic DNA and cloned into the 5' *NcoI* and 3' *NotI* restriction sites of pET28b(+) vector (Novagen, UK) for further His-Tag purification. *E. coli* DH5 $\alpha$  were transformed with the plasmid constructs for stable storage and *E. coli* BL21(DE3)pLysS (Promega, WI, USA) were used for expression and purification (Table S1).

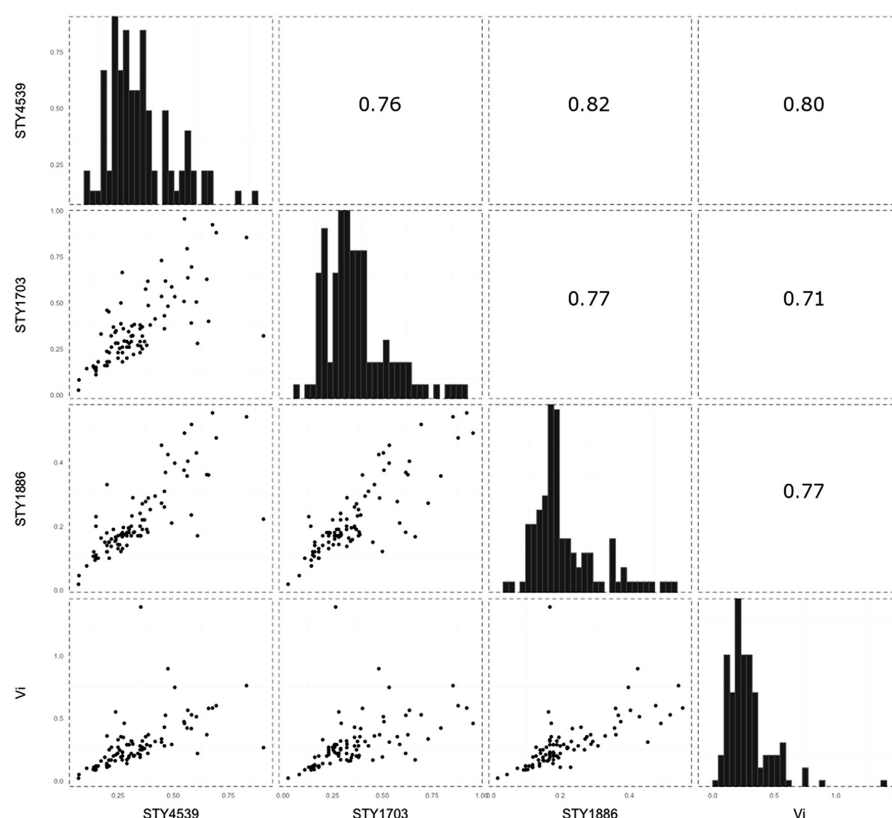
For protein expression, the *E. coli* BL21(DE3)pLysS strains harboring unique plasmid constructs (pEK90-pEK109) containing the genes of interest were inoculated into LB broth containing 100 mg/L kanamycin (Sigma, MO, USA), and incubated at  $37^{\circ}\text{C}$  overnight. Overnight cultures were diluted (1:100) into LB broth and incubated at  $37^{\circ}\text{C}$  with agitation until optical density ( $\text{OD}_{600}$ ) of 0.5. Expression of the exogenous proteins was induced by the addition of isopropyl- $\beta$ -D-thiogalactoside (IPTG) (Sigma-Aldrich, UK), to a final concentration of 0.1 mM. Bacterial cells

**Table 1** *Salmonella Typhi* antigens expressed in this study for serological testing.

Gene ID number <sup>a</sup>	Isotype detected using array <sup>b</sup>	Gene name	Amino acid identity <i>S. Typhi</i> CT18/ <i>S. Paratyphi</i> A AKU_12601	Annotation
STY0452	IgM	<i>yajI</i>	163/165 (98%)	Putative lipoprotein – Prokaryotic homolog of protein DJ-1
STY0796	IgM	<i>ybgF</i>	260/262 (99%)	Putative exported protein – Tol/pal system protein
STY1086	IgM	–	187/187 (100%)	Putative lipoprotein
STY1372	IgM	<i>pspB</i>	74/74 (100%)	Phage shock protein B
STY1612	IgM	–	Absent	Putative membrane protein – prophage associated
STY4539	IgM	<i>pilL</i>	Absent	Putative exported protein – type IV pili
STY1522	IgG	–	292/296 (99%)	Putative secreted protein – Choloylglycine secreted homolog
STY1703	IgG	<i>ssaP</i>	123/124 (99%)	Putative secreted protein – T3SS
STY1767	IgG	<i>nlpC</i>	48/140 (34%)	Putative lipoprotein – Endopeptidase
STY1886	IgG	<i>cdtB</i>	268/269 (99%)	Cytolethal distending toxin subunit B homolog
STY3208	IgG	–	277/279 (99%)	Hypothetical protein – Unknown function
STY4190	IgG	<i>yhjJ</i>	190/192 (99.0%)	Putative Zinc-protease

<sup>a</sup> Parkhill et al. 2001.<sup>48</sup>

<sup>b</sup> Liang et al. 2013.<sup>22</sup>



**Figure 1** Correlation of IgM measurements between selected *Salmonella* Typhi antigens. A representative selection of data showing a correlation in IgM measurements in human plasma for the antigens encoded by STY4539, STY1703, STY1886, and the Vi antigen. Histograms show the distribution of IgM levels by optical density to the highlighted *S. Typhi* antigen. The scatterplots below the histograms plot the IgM measurements of the two antigens on a right angle to the histograms and describe the correlation between antibody responses to two selected antigens. The numerals above the histograms depict the Spearman correlation coefficient ( $\rho$ ) values of the mirrored scatterplot. All other correlations are shown in [Fig. S1](#).

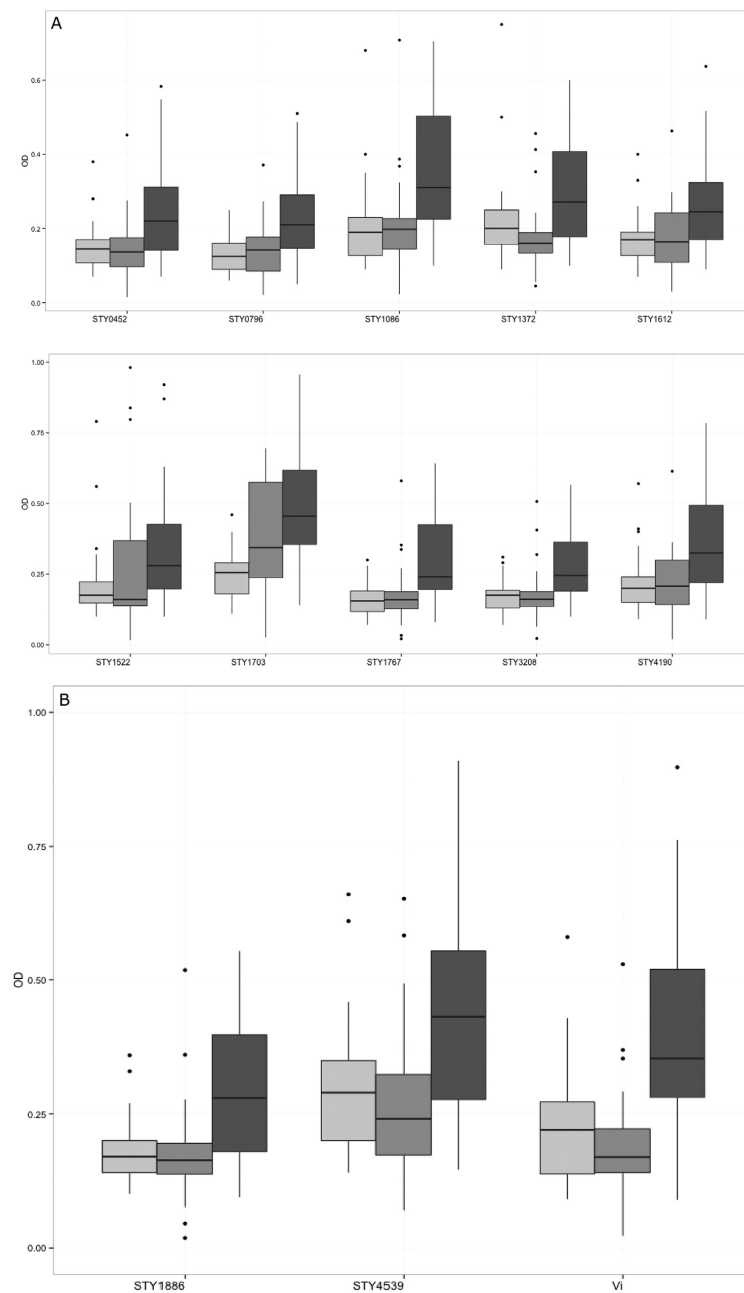
were harvested ( $5000 \times g$  at  $4^\circ\text{C}$  for 10 min) after 3 h of incubation at  $24^\circ\text{C}$ .

For soluble proteins, bacterial pellets were resuspended in 50 mM phosphate buffer (pH 8) containing 300 mM NaCl and 10 mM imidazole. After sonication, cell debris and the membrane fragment were pelleted by centrifugation at  $16,000 \times g$  at  $4^\circ\text{C}$  for 30 min. Supernatants were filtered through a  $0.45 \mu\text{m}$  membrane before being rocked at  $4^\circ\text{C}$  with nickel coated agarose beads (Ni-NTA, Invitrogen) for 2 h. Protein bound Ni-NTA beads were loaded into gravity flow columns (Qiagen, Germany) and washed with 20 mM imidazole in phosphate buffer. Proteins were eluted with 250 mM imidazole in phosphate buffer. For insoluble proteins a denaturing protocol was performed by firstly incubating the bacterial cells in an 8 M urea (pH 7.8) solution containing 20 mM sodium phosphate and 500 mM NaCl. Proteins were eluted with 4 M Urea (pH3) in a solution

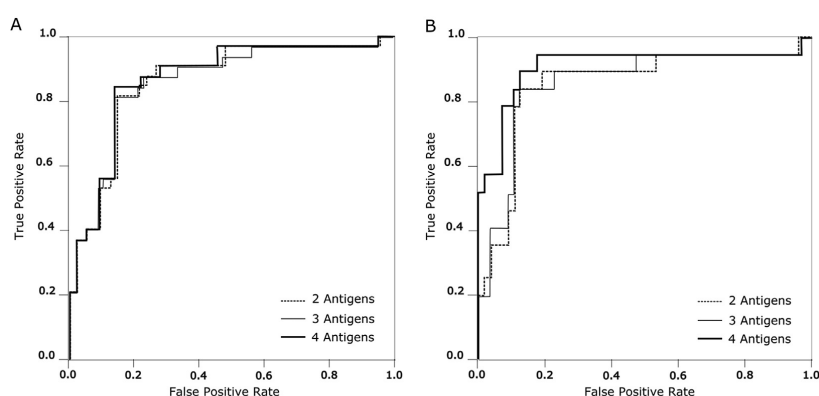
containing 20 mM Sodium Phosphate buffer and 500 mM NaCl. Proteins were renatured after purification in 50 mM Sodium Phosphate solution and 500 mM NaCl.

#### ELISAs using *S. Typhi* protein antigens

ELISAs to detect antigen specific IgM in human plasma samples were performed as described previously with 12 purified protein antigens and *S. Typhi* Vi polysaccharide antigen.<sup>24,25</sup> Briefly, 96 well flat-bottom ELISA plates (Nunc 2404, Thermo Scientific) were coated overnight with  $100 \mu\text{l}$  per well of the various antigens (final concentrations;  $7 \mu\text{g/ml}$  of protein antigens and  $1 \mu\text{g/ml}$  for the Vi antigen in 50 mM Carbonate Bicarbonate buffer). Coated plates were washed and blocked with 5% milk solution in PBS. After 2 h of blocking, plates were washed and incubated with



**Figure 2** IgM responses against *Salmonella* Typhi antigens in a Bangladeshi cohort of febrile patients and controls. Boxplots showing IgM measurements (optical density) in plasma from afebrile controls (light gray), febrile patients with an infection other than typhoid fever (medium gray), and confirmed typhoid patients (dark gray). Dark horizontal lines represent the mean IgM



**Figure 3** Assessing the sensitivity and specificity of IgM against *Salmonella* Typhi antigens for the diagnosis of typhoid fever. Receiver operating characteristic (ROC) curves summarizing the antibody responses against antigen combinations for the diagnosis of typhoid. The x-axis displays the false positivity rate (Specificity) and the y-axis displays true positive rate (Sensitivity). The performance of two, three and four antigens are shown by the dashed, gray, and black lines, respectively. A) ROC curve produced when the positive references are typhoid cases confirmed by blood culture and PCR amplification ( $n = 32$ ). B) ROC curve produced when the positive references are typhoid cases confirmed by blood culture only ( $n = 19$ ).

100  $\mu$ l (per well) of a 1:200 dilution of plasma at ambient temperature for 2 h. Plates were washed again and incubated with 100  $\mu$ l per well of alkaline phosphatase-conjugated anti-human IgM at ambient temperature for 1 h. The final ELISA plates were developed using p-Nitrophenyl phosphate (SigmaFAST N1891, Sigma–Aldrich, UK) substrate for 30 min at ambient temperature and the final absorbance were read at dual wavelengths (405 nm and 490 nm) using an automated microplate reader (Biorad). End point positive absorbance results were defined as optical densities (OD) greater than the absorbance obtained for the blank control wells plus four times the standard deviation. Three wells of culture *S. Typhi* positive plasma were run as control for every 96-well plate for each antigen. The results of each ELISA plate were accepted only if the OD values of the controlled were within their range of their known values plus/minus two standard deviations of the blank wells.

#### Statistical analysis

A geometric mean optical density was calculated to summarize the IgM response to the *S. Typhi* antigens in each arm of validation group including the negative reference population samples (healthy controls and other confirmed febrile infections) and the positive reference population (febrile patients with confirmed *S. Typhi*). The Wilcoxon

signed-rank test was used to test the null hypothesis; no difference in optical densities between the patient groups. Spearman's rho was used to investigate potential correlations between IgM antibody responses against the various antigens. Receiver operating characteristic (ROC) curves were used to determine the optimal cut-off and the specificity and sensitivity of the various antigens. A performance estimation of more than one antigen combination was evaluated using Support Vector Machine (SVM). All analyses were performed with R software (version 3.3.1; R Foundation for Statistical Computing). All confidence intervals (CIs) are reported 2-sided at the 95% intervals; all other significant testing were performed 2-sided with a significance level of  $p < 0.05$ .

## Results

### Acute IgM antibody responses against *Salmonella* Typhi antigens

Of the 18 protein antigens we targeted to purify, we were able to express and purify 12 (Table 1).

We firstly performed ELISAs independently employing the purified protein antigens and the Vi polysaccharide to detect IgM in the plasma of 40 healthy control subjects, 17 febrile individuals with a confirmed infection other than

measurement, with the box representing the 25th and 75th percentiles, whiskers represent the 5th and 95th percentiles; outliers are represented by dots. A) Boxplots of antibody responses against (from left to right and upper to lower) STY0452, STY0769, STY1086, STY1372, STY1612, STY1522, STY1703, STY1767, STY3208, and STY4190. All mean antibody measurements were statistically significant between the healthy controls and typhoid infections and between other infections and typhoid infections, with the exception of STY1522 ( $p < 0.05$  Wilcoxon Rank sum). B) Boxplots of antibody responses against (in order) STY4539, STY1886, Vi. All mean antibody measurements are statistically significant between the healthy controls and typhoid infections and between other infections and typhoid infections ( $p < 0.05$  Wilcoxon Rank sum).

typhoid, and 32 individuals with either blood culture or PCR (or both) confirmed typhoid infections ( $n = 89$  samples). We measured detectable IgM against all twelve of the purified *S. Typhi* proteins and the Vi polysaccharide in all of the 89 samples subjected to ELISA. We compared the IgM titers from each of the antigens individually to assess the performance of the antigens and to identify potential correlations between the serological targets. We found that early IgM responses against the majority of the novel *S. Typhi* protein antigens, with the exception of STY1522 ( $\rho < 0.7$ ), were highly correlated with one other ( $\rho > 0.8$ ). Notably, the IgM response to the protein antigens correlated weakly with those directed against the Vi polysaccharide ( $\rho < 0.6$ ) (Fig. 1 and Fig. S1).

### The diagnostic potential of IgM against *Salmonella Typhi* antigens

IgM against all twelve of the protein antigens and the Vi polysaccharide was significantly elevated in the plasma of the typhoid patients in comparison with the healthy controls ( $p < 0.05$ ) (Fig. 2). Furthermore, there was a significant differentiation in the plasma IgM measurements between the typhoid patients and those with a febrile disease with an alternative confirmed etiology with all antigens with the exception of STY1522 ( $p < 0.05$ ).

By assessing raw antibody measurements, we surmised that the best performing antigens, with respect to differentiating between the patient groups were STY4539, STY1886, and Vi (Fig. 2). The mean IgM responses (OD values) in the afebrile controls, the other confirmed infections and the typhoid infections were 0.29, 0.27, and 0.42 (against STY4539), 0.17, 0.18, and 0.25 (against STY1886), and 0.22, 0.21, and 0.35 (against Vi), respectively. This segregation between the patient groups was highly significant, resulting in  $p$ -values of 0.0001 and 0.003 for IgM against STY4539;  $<0.0001$  and 0.004 for IgM against STY1886; and 0.0001 and 0.0001 for IgM against Vi, between healthy controls and typhoid infections and between other febrile diseases and typhoid infections, respectively (Wilcoxon signed-rank test).

### Sensitivity and specificity of the serodiagnostic antigens

To further assess the IgM responses against the various *S. Typhi* antigens for the purposes of diagnostic testing we calculated sensitivities and specificities using a validation group incorporating two positive reference groups and a negative reference group for additional statistical power. The negative reference group ( $n = 57$ ) was the combination of data from the afebrile controls ( $n = 40$ ) and from those with a confirmed diagnosis other than typhoid ( $n = 17$  cases). The assay results were validated independently with two sets of positive reference data; these were a combination of blood culture confirmed *S. Typhi* along with those with a positive PCR amplification result for *S. Typhi* from blood ( $n = 32$ ), and the blood culture confirmed *S. Typhi* patients only ( $n = 19$ ).

The IgM responses against each of the antigens generated a continuous data set that was used to generate ROC

curves to optimize the index cut-off value. The defined cut-off values of the thirteen antigens corresponded with a range of sensitivities ranging from 0.50 to 0.84 and specificities between 0.58 and 0.84; areas under the ROC curve (AUC) ranged from 0.7 to 0.85. When used alone none of the antigens demonstrated a sensitivity or specificity  $>0.8$ . As predicted, Vi, STY4359, and STY1886 were the three antigens with the greatest serodiagnostic capacity in discriminating typhoid cases from afebrile controls and other infections. The sensitivities and specificities for identifying typhoid patients by IgM titers were 0.68; 0.8 (Vi), 0.62; 0.82 (STY4539), and 0.62; 0.82 (STY1886), respectively. Correspondingly, the AUCs were 0.84 (95%CI: 0.71, 0.96), 0.77 (95%CI: 0.61, 0.92), and 0.77 (95%CI: 0.62, 0.91).

We next employed SVM to identify combinations of two or more antigens across all 13 antigens to increase overall sensitivity and specificity. Using confirmed *S. Typhi* infection by blood culture or PCR as the positive reference ( $n = 32$ ), we found 11 combinations of two to four antigens that gave sensitivities from 0.81 to 0.84, specificities from 0.81 to 0.86, and AUCs from 0.87 to 0.87 (Fig. 3). We additionally identified 17 combinations of two to four antigens when using the positive reference as culture confirmed *S. Typhi* only ( $n = 19$ ), obtaining sensitivities from 0.84 to 0.89, specificities from 0.88 to 0.94, and AUCs from 0.859 to 0.912 (Fig. 3, Tables 2 and 3).

For the positive reference sets, IgM against Vi contributed to all of the combinations, while STY1703, STY1886, and STY4539 were present in more than half of the combinations. The remaining nine antigens contributed to at least one combination that gave sensitivities and specificities  $>0.8$ . These results demonstrated that, in the majority of examples, a combination of up to four antigens was directly associated with an increased performance of the IgM serology. However, the best performing antigens for the identification of typhoid patients by IgM were Vi in combination with either STY1703 or STY1886 (Tables 2 and 3).

### Identifying typhoid cases in patients with undiagnosed febrile disease

In this Bangladeshi cohort there were 226 patients with febrile disease without laboratory confirmed etiology and 18 with clinically suspected typhoid that were negative by blood culture and PCR amplification. We aimed to estimate the proportion of patients in this population who may have typhoid by applying the SVM cutoffs and combining the IgM titers against two *S. Typhi* antigens. We performed two independent analyses; the first combined IgM titers against STY1703 and Vi using a combination of culture confirmed *S. Typhi* and positive PCR amplification for *S. Typhi* from blood as the positive reference group (Fig. 4 and Table 3). Using these criteria we found that 142/226 (59%) of the undiagnosed febrile patient group and 16/18 (88%) with clinically suspected typhoid had IgM titers indicative of typhoid. Using more stringent criteria (blood culture confirmed patients) as the positive reference and a combination of IgM against Vi and STY1886 we found that 119/226 (52%) febrile



**Table 2** The sensitivity and specificity of multiple antigens for typhoid diagnosis using blood culture and PCR positive patients as positive reference group (n = 32).

Antigen combinations	STY1703 & Vi	STY4539 & STY1703 & Vi	STY4539 & STY1703 & STY1886 & Vi
Specificity	0.86	0.86	0.86
Sensitivity	0.81	0.84	0.84
Number with undiagnosed febrile disease predicted to have typhoid (n = 243)	135 (59%)	134 (59%)	134 (59%)
Number with clinically suspected typhoid disease predicted to have typhoid (n = 18)	16 (88%)	16 (88%)	16 (88%)
AUC (95%CI)	0.865 (0.782, 0.947)	0.863 (0.779, 0.947)	0.866 (0.783, 0.949)

**Table 3** The sensitivity and specificity of multiple antigens for typhoid diagnosis using blood culture positive patients only as positive reference group (n = 19).

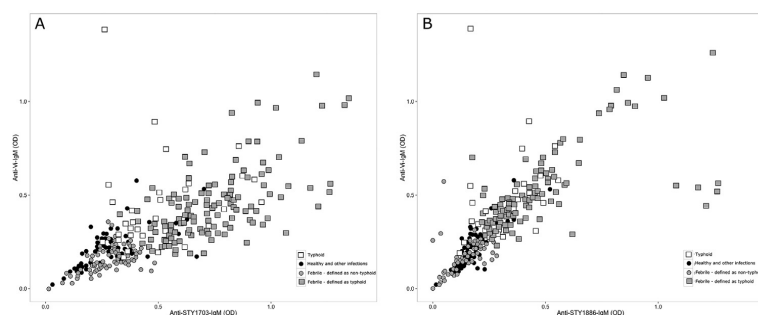
Antigen combinations	STY1886 & Vi	STY4190 & STY1886 & Vi	STY4539 & STY4190 & STY1886 & Vi
Specificity	0.88	0.89	0.88
Sensitivity	0.84	0.84	0.89
Number with undiagnosed febrile disease predicted to have typhoid (n = 226)	119 (52%)	78 (34%)	71 (31%)
Number with clinically suspected typhoid disease predicted to have typhoid (n = 18)	15 (83%)	13 (72%)	13 (72%)
AUC (95%CI)	0.859 (0.746, 0.972)	0.891 (0.791, 0.991)	0.912 (0.81, 1.014)

cases and 15/18 (83%) clinically suspected typhoid, respectively, had a profile indicative of typhoid (Fig. 4).

## Discussion

Typhoid is caused by a human restricted pathogen that is transmitted fecal-orally. Consequently, improving the

quality of drinking water supplies and education in better hygiene practices are likely the most effective measures to for typhoid elimination. However, these interventions cannot be promptly realized in the endemic areas of Africa and South Asia. Therefore, the short-term control of typhoid is dependent on large vaccination programmes and appropriate treatment, both of which, for differing



**Figure 4** Detecting febrile patients with an IgM profile indicative of typhoid fever. Plots predicting the number of undiagnosed febrile patients that have an IgM measurement indicative of typhoid fever. The black circles represent the negative controls, which includes healthy controls and patients other with infections. The white boxes represent typhoid cases confirmed by blood culture or PCR. The gray circles are febrile patients with an IgM profile indicative of not having typhoid fever, gray boxes are febrile patients defined as having a typhoid infection using the pre-defined IgM profile. A) Plot where the positive reference was defined as the typhoid cases confirmed by blood culture or PCR (n = 32); the selected antigen combination was STY1703 and Vi. B) Plot where the positive reference was defined as the typhoid cases confirmed by blood culture only (n = 19); the selected antigen combination was STY1886 and Vi.



reasons, rely on better case detection. The fact that there is not currently a typhoid diagnostic assay with a high degree of sensitivity or specificity limits disease burden assessments,<sup>26,27</sup> and may result in patients being misdiagnosed and receiving a sub-optimal therapy.<sup>28</sup> Furthermore, with the global increase in *S. Typhi* associated with reduced susceptibility and resistance against the fluoroquinolones and other antimicrobials,<sup>29,30</sup> the demand for typhoid diagnostics are now greater than ever.<sup>31,32</sup>

There is a paucity of data arising from studies on humans with typhoid that have measured the immunological response to *S. Typhi* specific antigens.<sup>7</sup> Studies that have been performed have found significant interferon- $\gamma$  (IFN- $\gamma$ ) responses in cells stimulated with various antigens including fimbriae and outer membrane proteins using polymorphonuclear cells from the blood of typhoid patients.<sup>33</sup> Further, when whole blood from typhoid patients is stimulated with *S. Typhi* lipopolysaccharide, TNF $\alpha$  release is lower during active typhoid than after antimicrobial treatment, indicating a short immune modulation effect, which may be induced by the Vi polysaccharide.<sup>34</sup> Antigen arrays and other probing techniques have been used successfully to interrogate the antibody repertoire during early infection,<sup>22,35–37</sup> and have detected antibody responses to several novel, and potentially organism specific, antigens that may be able to distinguish typhoid patients from controls.<sup>22</sup> Using this “screening data” we rationally selected several *S. Typhi* protein antigens and aimed to investigate if early immunological diagnostic signals could be detected in the plasma of febrile patients.

Our study focused on a well-defined patient group from Bangladesh, who were enrolled for the primary focus of studying typhoid diagnostics. Whilst the patient numbers with typhoid in this group were relatively modest, the clinical and laboratory criteria for patients with febrile disease were consistent and have been previously assessed with commercial serological tests for typhoid.<sup>23</sup> We noted that the IgM response against the twelve purified *S. Typhi* protein antigens were stable and well correlated; the IgM responses in comparison to Vi correlated less consistently. These data confirm our original antigen screening data and suggest that these antigens are immunogenic and induce an antibody response early in infection. Given the poor performance of commercial RDTs,<sup>38,39</sup> our data signify that the early detection of IgM against more specific *S. Typhi* protein antigens may be a more specific and sensitive approach for developing a RDT for typhoid.

Indistinguishable clinical features and the lack of a reliable gold standard test complicate typhoid diagnosis. Here, the IgM response against all 12 antigens was significantly higher in typhoid patients than both afebrile controls and patients with febrile diseases other than typhoid. Furthermore, through inference from the AUC under the ROC curve we were able to identify the best three performing antigens, which were encoded by STY4539 (PiL) and STY1886 (CdtB) in combination with the Vi polysaccharide. PiL is a putative exported protein and a component of the type IV pili encoded adjacent to the genes encoding Vi on SPI-7.<sup>40,41</sup> The PiL protein is induced following uptake by human derived macrophages,<sup>42</sup> and the type IV pili to which it is associated facilitates entry into epithelial cells.<sup>43</sup> CdtB, encoded by STY1886, is one of

the two A sub-units of typhoid toxin, an AB type toxin.<sup>44</sup> Typhoid toxin is a virulence-associated factor of *S. Typhi*, which is thought to be associated with the early symptoms of typhoid.<sup>45</sup> We confirm that this component of typhoid toxin is immunogenic and may be an important biomarker of acute typhoid.<sup>22,46</sup> Whilst these three virulence factors (PiL, CdtB, and Vi) were not sufficient in themselves to produce a reproducibly high (>0.8) degree of sensitivity and specificity for typhoid diagnosis, we gained additional power by combining data from >1 antigen using an SVM model. The IgM responses against Vi in combination with either PiL or CdtB were found to generate the highest degree of sensitivity and specificity. Seemingly, a combination of the differing IgM responses against polysaccharide and a protein compensates for a lower affinity to one of the antigens. Furthermore, we were able to estimate the proportion of the population that may have typhoid by imposing cut-offs from the typhoid confirmed patients onto the population with undiagnosed febrile diseases. These data did not generate a precise cut-off, therefore, our data suggest that typhoid diagnostics are not an exact science and our data should be interpreted with caution. These methods warrant further investigation in additional cohorts, but it suggests a substantial burden of undiagnosed febrile disease is associated with *S. Typhi* in this setting.

This study has limitations. The sample size was relatively small; we aimed to rectify this by including a subset of patients that appear to have *S. Typhi* DNA in their bloodstream but were culture negative.<sup>47</sup> Using this combination of methods as a gold standard we were able to increase the diagnostic power of the assays. A further limitation is that this study was conducted in a single healthcare location over a limited time period. Whilst our data provide some confidence that these serological assays may be of utility for typhoid diagnostics, these methods should be validated in additional cohorts. However, there remains a challenge in identifying typhoid patients that have a sterile blood culture; a combination of novel approaches, such as metabolomics and/or functional genomics,<sup>17,21</sup> in a febrile disease cohort may add further insight into this important patient group.

In conclusion, we have investigated the serological diagnostic potential of *S. Typhi* protein antigens and the Vi polysaccharide in a group of patients with febrile diseases in Bangladesh. Our novel data show that serology may have some utility for typhoid diagnostics and a combination of antigens improves the diagnostic potential. Our assays give high levels of sensitivity and specificity, but require further assessment in differing patient populations.

## Conflict of interest

The authors declare no competing interests.

## Funding sources

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data collection and analysis, decision to publish, or preparation of the manuscript.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.jinf.2017.05.007>.

## References

- Ochiai RL, Acosta CJ, Danovaro-holliday MC, Baiqing D, Bhattacharya SK, Agtini MD, et al. A study of typhoid fever in five Asian countries: disease burden and implications for controls. *Bull World Health Organ* 2008;39818. <http://dx.doi.org/10.2471/BLT.06.039818>.
- Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. Typhoid fever. *N Engl J Med* 2002;347:1770–82. <http://dx.doi.org/10.1056/NEJMra020201>.
- Crump JA, Mintz ED. Global trends in typhoid and paratyphoid fever. *Clin Infect Dis* 2010;50:241–6. <http://dx.doi.org/10.1086/649541>.
- Mogasale V, Maskery B, Ochiai RL, Lee JS, Mogasale VV, Ramani E, et al. Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment. *Lancet Glob Heal* 2014;2:e570–80. [http://dx.doi.org/10.1016/S2214-109X\(14\)70301-8](http://dx.doi.org/10.1016/S2214-109X(14)70301-8).
- Karkey A, Jombart T, Walker AW, Thompson CN, Torres A, Dongol S, et al. The ecological dynamics of fecal contamination and *Salmonella* Typhi and *Salmonella* Paratyphi A in municipal Kathmandu drinking water. *PLoS Negl Trop Dis* 2016;10:e0004346. <http://dx.doi.org/10.1371/journal.pntd.0004346>.
- Woods CW, Murdoch DR, Zimmerman MD, Glover W a, Basnyat B, Wolf L, et al. Emergence of *Salmonella enterica* serotype Paratyphi A as a major cause of enteric fever in Kathmandu, Nepal. *Trans R Soc Trop Med Hyg* 2006;100:1063–7. <http://dx.doi.org/10.1016/j.trstmh.2005.12.011>.
- Dougan G, Baker S. *Salmonella enterica* serovar Typhi and the pathogenesis of typhoid fever. *Annu Rev Microbiol* 2014;68:317–36. <http://dx.doi.org/10.1146/annurev-micro-091313-103739>.
- Nga TVT, Parry CM, Le T, Lan NPH, Diep TS, Campbell JI, et al. The decline of typhoid and the rise of non-typhoid salmonellae and fungal infections in a changing HIV landscape: bloodstream infection trends over 15 years in southern Vietnam. *Trans R Soc Trop Med Hyg* 2012;106:26–34. <http://dx.doi.org/10.1016/j.trstmh.2011.10.004>.
- Kiratisin P. Bacteraemia due to non-typhoidal *Salmonella* in Thailand: clinical and microbiological analysis. *Trans R Soc Trop Med Hyg* 2008;102:384–8. <http://dx.doi.org/10.1016/j.trstmh.2008.01.019>.
- Crump JA. Building the case for wider use of typhoid vaccines. *Vaccine* 2015;33:C1–2.
- Baker S, Favorov M, Dougan G. Searching for the elusive typhoid diagnostic. *BMC Infect Dis* 2010;10:45. <http://dx.doi.org/10.1186/1471-2334-10-45>.
- Parry CM, Wijedoru L, Arjyal A, Baker S. The utility of diagnostic tests for enteric fever in endemic locations. *Expert Rev Anti Infect Ther* 2011;9:711–25. <http://dx.doi.org/10.1586/eri.11.47>.
- Gilman RH, Termini M, Levine MM, Hernandez-Mendoza P, Hornick RB. Relative efficacy of blood, urine, rectal swab, bone-marrow, and rose-spot cultures for recovery of *Salmonella* Typhi in typhoid fever. *Lancet* 1975;1:1211–3.
- Wain J, Bay PVANBE, Vinh HA, Duong NM, Diep TOS, Walsh AL, et al. Quantitation of bacteria in bone marrow from patients with typhoid fever: relationship between counts and clinical features. *Society* 2001;39:1571–6. <http://dx.doi.org/10.1128/JCM.39.4.1571>.
- Nga TVT, Karkey A, Dongol S, Thuy HN, Dunstan S, Holt K, et al. The sensitivity of real-time PCR amplification targeting invasive *Salmonella* serovars in biological specimens. *BMC Infect Dis* 2010;10:125. <http://dx.doi.org/10.1186/1471-2334-10-125>.
- Waddington CS, Darton TC, Jones C, Haworth K, Peters A, John T, et al. An outpatient, ambulant-design, controlled human infection model using escalating doses of *Salmonella* Typhi challenge delivered in sodium bicarbonate solution. *Clin Infect Dis* 2014;58:1230–40. <http://dx.doi.org/10.1093/cid/ciu078>.
- Blohmke CJ, Darton TC, Jones C, Suarez NM, Waddington CS, Angus B, et al. Interferon-driven alterations of the host's amino acid metabolism in the pathogenesis of typhoid fever. *J Exp Med* 2016;213:1061–77. <http://dx.doi.org/10.1084/jem.20151025>.
- Darton TC, Blohmke CJ, Giannoulou E, Waddington CS, Jones C, Sturges P, et al. Rapidly escalating hepcidin and associated serum iron starvation are features of the acute response to typhoid infection in humans. *PLoS Negl Trop Dis* 2015;9:e0004029. <http://dx.doi.org/10.1371/journal.pntd.0004029>.
- Kantele A, Pakkanen SH, Karttunen R, Kantele JM. Head-to-head comparison of humoral immune responses to Vi capsular polysaccharide and *Salmonella* Typhi Ty21a typhoid vaccines—a randomized trial. *PLoS One* 2013;8:e60583. <http://dx.doi.org/10.1371/journal.pone.0060583>.
- Charles RC, Liang L, Khanam F, Sayeed MA, Hung C, Leung DT, et al. Immunoproteomic analysis of antibody-in-lymphocyte supernatant (ALS) in patients with typhoid fever in Bangladesh. *Clin Vaccine Immunol* 2013. <http://dx.doi.org/10.1128/CVI.00661-13>.
- Näsström E, Thieu Nga TV, Dongol S, Karkey A, Voong Vinh P, Ha Thanh T, et al. *Salmonella* Typhi and *Salmonella* Paratyphi A elaborate distinct systemic metabolite signatures during enteric fever. *Elife* 2014:e03100. <http://dx.doi.org/10.7554/eLife.03100>.
- Liang L, Juarez S, Nga TVT, Dunstan S, Nakajima-Sasaki R, Davies DH, et al. Immune profiling with a *Salmonella* Typhi antigen microarray identifies new diagnostic biomarkers of human typhoid. *Sci Rep* 2013;3:1043. <http://dx.doi.org/10.1038/srep01043>.
- Maude RR, de Jong HK, Wijedoru L, Fukushima M, Ghose A, Samad R, et al. The diagnostic accuracy of three rapid diagnostic tests for typhoid fever at Chittagong Medical College Hospital, Chittagong. *Bangladesh Trop Med Int Health* 2015;20:1376–84. <http://dx.doi.org/10.1111/tmi.12559>.
- Karkey A, Thompson CN, Tran Vu Thieu N, Dongol S, Le Thi Phuong T, Voong Vinh P, et al. Differential epidemiology of *Salmonella* Typhi and Paratyphi A in Kathmandu, Nepal: a matched case control investigation in a highly endemic enteric fever setting. *PLoS Negl Trop Dis* 2013;7:e2391. <http://dx.doi.org/10.1371/journal.pntd.0002391>.
- Rondini S, Micoli F, Lanzilao L, Pisoni I, Di Ciccio V, Saul AJ, et al. Characterization of *Citrobacter* sp. line 328 as a source of Vi for a Vi-CRM197 glycoconjugate vaccine against *Salmonella* Typhi. *J Infect Dev Ctries* 2012;6:763–73. <http://dx.doi.org/10.3855/jidc.2495>.
- Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World Health Organ* 2004;82:346–53.
- Bhutta ZA. Current concepts in the diagnosis and treatment of typhoid fever. *BMJ* 2006;333:78–82. <http://dx.doi.org/10.1136/bmj.333.7558.78>.
- Arjyal A, Basnyat B, Nhan HT, Koirala S, Giri A, Joshi N, et al. Gatifloxacin versus ceftriaxone for uncomplicated enteric

- fever in Nepal: an open-label, two-centre, randomised controlled trial. *Lancet Infect Dis* 2016. [http://dx.doi.org/10.1016/S1473-3099\(15\)00530-7](http://dx.doi.org/10.1016/S1473-3099(15)00530-7).
29. Pham Thanh D, Karkey A, Dongol S, Ho Thi N, Thompson CN, Rabaa MA, et al. A novel ciprofloxacin-resistant subclade of H58 *Salmonella* Typhi is associated with fluoroquinolone treatment failure. *Elife* 2016;5. <http://dx.doi.org/10.7554/eLife.14003>.
  30. Wong VK, Baker S, Pickard DJ, Parkhill J, Page AJ, Feasey NA, et al. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of *Salmonella* Typhi identifies inter- and intracontinental transmission events. *Nat Genet* 2015;47:632–9. <http://dx.doi.org/10.1038/ng.3281>.
  31. Steele AD, Hay Burgess DC, Diaz Z, Carey ME, Zaidi AKM. Challenges and opportunities for typhoid fever control: a call for coordinated action. *Clin Infect Dis* 2016;62(Suppl 1):S4–8. <http://dx.doi.org/10.1093/cid/civ976>.
  32. Andrews JR, Ryan ET. Diagnostics for invasive *Salmonella* infections: current challenges and future directions. *Vaccine* 2015;33(Suppl 3):C8–15. <http://dx.doi.org/10.1016/j.vaccine.2015.02.030>.
  33. Sheikh A, Khanam F, Sayeed MA, Rahman T, Pacek M, Hu Y, et al. Interferon- $\gamma$  and proliferation responses to *Salmonella enterica* serotype Typhi proteins in patients with S. Typhi bacteremia in Dhaka, Bangladesh. *PLoS Negl Trop Dis* 2011;5. <http://dx.doi.org/10.1371/journal.pntd.0001193>. e1193.
  34. Raffatellu M, Santos RL, Chessa D, Wilson RP, Winter SE, Rossetti CA, et al. The capsule encoding the *viaB* locus reduces interleukin-17 expression and mucosal innate responses in the bovine intestinal mucosa during infection with *Salmonella enterica* serotype Typhi. *Infect Immun* 2007;75:4342–50. <http://dx.doi.org/10.1128/IAI.01571-06>.
  35. Sheikh A, Charles RC, Sharmeen N, Rollins SM, Harris JB, Bhuiyan MS, et al. In vivo expression of *Salmonella enterica* serotype Typhi genes in the blood of patients with typhoid fever in Bangladesh. *PLoS Negl Trop Dis* 2011;5. <http://dx.doi.org/10.1371/journal.pntd.0001419>. e1419.
  36. Charles RC, Sultana T, Alam MM, Yu Y, Wu-Freeman Y, Bufano MK, et al. Identification of immunogenic *Salmonella enterica* serotype Typhi antigens expressed in chronic biliary carriers of S. Typhi in Kathmandu, Nepal. *PLoS Negl Trop Dis* 2013;7. <http://dx.doi.org/10.1371/journal.pntd.0002335>. e2335.
  37. Harris JB, Baresch-Bernal A, Rollins SM, Alam A, LaRocque RC, Bikowski M, et al. Identification of in vivo-induced bacterial protein antigens during human infection with *Salmonella enterica* serovar Typhi. *Infect Immun* 2006;74:5161–8. <http://dx.doi.org/10.1128/IAI.00488-06>.
  38. Moore CE, Pan-Ngum W, Wijedoru LPM, Sona S, Nga TVT, Duy PT, et al. Evaluation of the diagnostic accuracy of a typhoid IgM flow assay for the diagnosis of typhoid fever in Cambodian children using a Bayesian latent class model assuming an imperfect gold standard. *Am J Trop Med Hyg* 2014;90:114–20. <http://dx.doi.org/10.4269/ajtmh.13-0384>.
  39. Islam K, Sayeed MA, Hossen E, Khanam F, Charles RC, Andrews J, et al. Comparison of the performance of the TPTest, tubex, typhidot and Widal immunodiagnostic assays and blood cultures in detecting patients with typhoid fever in Bangladesh, including using a Bayesian latent class modeling approach. *PLoS Negl Trop Dis* 2016;10:e0004558. <http://dx.doi.org/10.1371/journal.pntd.0004558>.
  40. Pickard D, Wain J, Baker S, Line A, Chohan S, Fookes M, et al. Composition, acquisition, and distribution of the Vi exopolysaccharide-encoding *Salmonella enterica* pathogenicity island SPI-7. *Society* 2003;185:5055–65. <http://dx.doi.org/10.1128/JB.185.17.5055>.
  41. Tsui ISM, Yip CMC, Hackett J, Morris C. The type IVB pili of *Salmonella enterica* serovar Typhi bind to the cystic fibrosis transmembrane conductance regulator. *Infect Immun* 2003;71:6049–50.
  42. Faucher SP, Curtiss R, Daigle F. Selective capture of *Salmonella enterica* serovar typhi genes expressed in macrophages that are absent from the *Salmonella enterica* serovar Typhimurium genome. *Infect Immun* 2005;73:5217–21. <http://dx.doi.org/10.1128/IAI.73.8.5217-5221.2005>.
  43. Zhang X, Tsui ISM, Yip CMC, Fung ADAWY, Wong DK, Dai X, et al. *Salmonella enterica* Serovar Typhi uses type IVB Pili to enter human intestinal epithelial cells. *Society* 2000;68:3067–73.
  44. Song J, Gao X, Galán JE. Structure and function of the *Salmonella* Typhi chimaeric A(2)B(5) typhoid toxin. *Nature* 2013;499:350–4. <http://dx.doi.org/10.1038/nature12377>.
  45. Galán JE. Typhoid toxin provides a window into typhoid fever and the biology of *Salmonella* Typhi. *Proc Natl Acad Sci U. S. A* 2016;113:6338–44. <http://dx.doi.org/10.1073/pnas.1606335113>.
  46. Charles RC, Sheikh A, Krastins B, Harris JB, Bhuiyan MS, LaRocque RC, et al. Characterization of anti-*Salmonella enterica* serotype Typhi antibody responses in bacteremic Bangladeshi patients by an immunoaffinity proteomics-based technology. *Clin Vaccine Immunol* 2010;17:1188–95. <http://dx.doi.org/10.1128/CVI.00104-10>.
  47. Wijedoru LPM, Kumar V, Chanpheaktra N, Chheng K, Smits HL, Pastoor R, et al. Typhoid fever among hospitalized febrile children in Siem Reap, Cambodia. *J Trop Pediatr* 2012;58:68–70. <http://dx.doi.org/10.1093/tropej/fmr032>.
  48. Parkhill J, Dougan G, James KD, Thomson NR, Pickard D, Wain J, et al. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature* 2001;413:848–52. <http://dx.doi.org/10.1038/35101607>.